EVALUATION OF DIRECT ANTIGLOBULIN TEST POSITIVE CASES BY ELUTION STUDY

Dissertation submitted to

THE TAMILNADU Dr.M.G.R. MEDICAL UNIVERSITY

In partial fulfilment of the regulations

For the award of the degree of

M.D BRANCH - XXI

IMMUNOHAEMATOLOGY &

BLOOD TRANSFUSION

DEPARTMENT OF TRANSFUSION MEDICINE

THE TAMILNADU Dr.M.G.R. MEDICAL UNIVERSITY

CHENNAI, INDIA

MAY 2019

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LIST OF ABBREVIATIONS

IHA	-	Immune Hemolytic Anemia
AIHA	-	Autoimmune Hemolytic Anemia
WAIHA	-	Warm Autoimmune Hemolytic Anemia
CAS	-	Cold Agglutinin Syndrome
PCH	-	Paroxysmal Cold Hemoglobinuria
mAIHA	-	Mixed Autoimmune Hemolytic Anemia
DIIHA	-	Drug Induced Immune Hemolytic Anemia
HDFN	-	Hemolytic Disease of Fetus and New-born
HTR	-	Hemolytic Transfusion Reactions
DDAB	-	Drug Dependent Antibody
DIAB	-	Drug Independent Antibodies
DAT	-	Direct Antiglobulin Test
IAT	-	Indirect Antiglobulin Test
Ig	-	Immunoglobulin
RBC	-	Red Blood Cell
CLL	-	Chronic Lymphocytic Leukemia
AHG	-	Anti Human Globin
SLE	-	Systematic Lupus Erythematosus
CTT	-	Conventional Tube Technique
GT	-	Gel Technique
PBS	-	Phosphate Buffer Saline
RT	-	Room Temperature
DTT	-	di-thiotheritol
2-ME	-	2-Mercaptoethanol
ET	-	Exchange Transfusion
LISS	-	Low Ionic Saline Suspension
EDTA	-	Ethylene Diamine Tetra Acetic Acid

INTRODUCTION

The direct antiglobulin test (DAT) is used to determine whether the red blood cells are coated in vivo with antibodies such as immunoglobulin, complement or both. The direct antiglobulin test is also referred as the Direct Coombs' test since it is based on the test developed by Coombs, Mourant and Race.[1]

Depending on the technique and the reagents used, a positive direct antiglobulin test has been reported in 1:1000 to 1:14,000 blood donors and 1% to 15% of hospital patients. The direct antiglobulin test is used most commonly to investigate possible haemolytic transfusion reactions, haemolytic disease of the fetus and newborn (HDFN), autoimmune haemolytic anaemia (primary or secondary), alloimmune haemolytic anaemia and drug induced immune haemolysis.[2] These coated red cells are difficult to cross match, which is required for selection of an appropriate unit of blood for transfusion in this patients.[3]

Clinical picture of WAIHA is highly variable. Most patients have symptoms correlated to anaemia, such as fatigue, palpitations and shortness of breath. Occasionally massive haemolysis manifested by haemoglobinuria, haemglobinemia and profound anaemia can be seen with secondary WAIHA.[4]

Direct antiglobulin test (DAT) is used to determine whether the red cells have been coated in vivo with IgG or complement or both. Stronger the DAT, the antibody is expected to cause more haemolysis if the antigen positive donor unit is transfused.[5] The DAT can be initially performed with a polyspecific antihuman globulin (AHG) reagent that is capable of detecting both IgG and C3d. If the results are positive, tests with monospecific reagents (anti-IgG and anti-complement) need to be performed to appropriately characterize the immune process involved and determine the diagnosis.[6] Then removal of antibodies from in vivo sensitized red

cells by elution technique to identify them. Various elution procedures are used for dissociating antibodies from red cells. In studies Elution removes antibody molecules from the red cell membrane either by disrupting the antigen or changing conditions to favour dissociation of antibody from antigen conducted for the efficacy of various elution methods viz., Acid elution, Glycine-HCl/EDTA, heat elution and Chloroquine diphosphate, and Cold elution, the Acid elution method is suitable for eluting auto and allo Antibodies present on the RBCs .[7]

Clinical signs and symptoms of immune haemolysis are present. Serum test results are negative or inconclusive for a patient who has been recently transfused. HDFN is suspected but no alloantibodies were detected in the maternal plasma.[8]

Usually the same specificity can be detected in the patient's (or, in HDN, the mother's) serum, eluate is of increased help in antibody identification when serum reactions are weak. When the eluate reacts with all cells tested, autoantibody is the most likely explanation, especially if the patient has not been recently transfused. When no unexpected antibodies are present in the serum, and if the patient has not been recently transfused, no further serologic testing of an isolated autoantibody is necessary. Sometimes no reactivity is detected in the eluate, despite reactivity of the cells with specific anti-IgG. The cause may be that the eluate was not tested against cells positive for the corresponding antigen, notably group A or group B cells.[9]

This cross sectional study is conducted to find out serological characterization of red cell bound antibodies with regard to antibody class, subclass, DAT strength and their correlation with in vivo haemolysis and also the effect of acid elution (glycine acid /Glycine acid EDTA) in DAT positive patients.

AIM AND OBJECTIVES

AIM

To determine class and subclass of red cell bound antibodies in Direct Antiglobulin Test positive cases and to perform elution studies to find out antibody specificity.

OBJECTIVE

1. To find the class and subclass of red cell bound antibodies thereby appropriately characterizing the immune process involved and determining the diagnosis.

2. To study the correlation between Direct Antiglobulin Test strength and in vivo haemolysis based on clinical parameters.

3. To perform elution studies in Direct Antiglobulin Test (IgG) positive cases and to determine antibody specificity.

4. To distinguish between auto antibody and alloantibody.

REVIEW OF LITERATURE

THE DIRECT ANTIGLOBULIN test (DAT) is a simple test used to determine if the red cells have been coated in vivo with immunoglobulin (Ig), complement, or both. The DAT is used primarily for the investigation of autoimmune hemolytic anemia (AIHA), drug-induced immune hemolysis, hemolytic disease of the fetus and new-born (HDFN) and hemolytic transfusion reactions,. A positive DAT result may or may not be associated with immune mediated hemolysis.[3]

HISTORY OF DAT

"The first lesson to be learned in history is that the path of process is anything but straight."

In the 1940s, the actual nature of antibodies was still unknown, but seemed to be associated with the serum globulins. Race, Mourant and Weiner concluded that there were two types of Rh antibody: one that bound to the RBC surface and caused agglutination (the "complete" antibody) and another that absorbed to the RBC surface but did not cause agglutination (the "incomplete" antibody).[1]

In 1945, Coombs, Mourant and Race described technique for detecting attachment of Rh antibodies in serum that did not produce agglutination. This test is known as the antiglobulin test (AHG) and uses antibody to human globulin. In 1946, Coombs and associates described the use of AHG to detect in vivo sensitization of the red cells of babies suffering from hemolytic disease of the new-born (HDN). Although the test was initially of great discovery in the investigation of Rh hemolytic disease of the new-born, it was not long before its use for detection of other IgG blood group antibodies became clearly evident. The first of the Kell blood

group system antibodies and its associated antigen were reported only weeks after Coombs had described the test.[1,2]

The principle of the instrumental in introducing the antiglobulin test to blood group serology had in fact been described by Moreschi in 1908 before Coombs and associates. The study of Moreschi involved the use of rabbit anti-goat serum to agglutinate rabbit red cells, which were sensitized with low no agglutinating does of goat anti-rabbit red cells serum. Coombs's production involved the injection rabbits with human serum to produce antihuman serum. The absorption is used to remove heterospecific antibodies and the dilution to avoid prozone but the antiglobulin serum still retained sufficient antibody activity to permit cross-linking of adjacent red cells coated with IgG antibodies.[1]

The antiglobulin test was first used to demonstrate antibody in serum, but later the same principle was used to demonstrate in-vivo sensitization of red cells with antibodies or complement components. As used in immunohematology, antiglobulin testing generates visible agglutination of sensitized red cells. An indirect antiglobulin test is used to demonstrate in-vitro reactions between red cells and antibodies that sensitize, but do not agglutinate, cells that express the corresponding antigen.[11]

STRUCTURE OF IMMUNOGLOBULINS

Immunoglobulin (Ig), is a complex protein produced by plasma cells, with specificity towards specific antigens. Each specific antigen stimulates the production of specific antibody. These antibodies, binds to the antigen, and/or fixes complement, facilitate phagocytosis, and neutralises toxic substances found in the circulation. Thus, antibodies have various functions, some type of Immunoglobulins are highly specialized and more specific than others.

Immunoglobulins are also called as antibodies; they are made up of four polypeptide chains-two identical heavy chains (H) and two identical light chains (L). Light chains have molecular weight of approximately 22,500 Daltons and heavy chains have molecular weight of 50,000-75,000 Daltons which are interconnected by covalent disulfide bonds. The heavy chains are held together by disulfide bonds at their hinge region. The H chains differ in structural and antigenic properties. These chains determine the class and subclass of the molecule.

Five different classes of Ig are recognized, IgG, IgM, IgA, IgD and IgE, and these have different H chains, termed gamma (γ), mu (μ), alpha (α), delta (δ) and epsilon (ε). Igs of all five classes have the same L chains, although these may be either kappa (κ) or lambda (λ). In each Immunoglobulin molecule, the two L chains are the same, e.g. a molecule may be γγκκ or γγλλ, it is the variation in the heavy chains makes difference in each type of immunoglobulins. IgG is the most common and concentrated in serum, consisting nearly 80% of the total serum immunoglobulin; second most common immunoglobulin is IgA, present as 13% of total immunoglobulins predominantly found in body secretions. IgM has a concentration of 6%; IgD is 1% and IgE is the least common immunoglobulin present less than 1%.[5] IgG molecules occur as monomers, IgM molecules as pentamers, e.g. (μ2λ2)5, and IgA molecules as monomers or dimers.

Immunoglobulins are protein molecules have two terminal regions, these are the amino (NH2) terminal and the carboxy (COOH) terminal. Amino terminal region consisting both light and heavy chains of immunoglobulin is known as the variable region. This variation in the structure is according to the great variation in antibody specificity and it is responsible for antigen binding. The carboxy terminal of all heavy chains has a constant aminoacid sequence and is named as constant region. The Fc region extends from the carboxy terminal to the hinge region and is

primarily responsible for monocyte binding and complement fixation on Fc receptors present on the cell. The Fab fragments extend from the amino terminal to the hinge region of the molecule.

In Immunoglobulin molecule the domains are made up of regions of both heavy and light chains, folded into globular structures or loops and these are made up of approximately 110-120 amino acids. The domains consist of variable (V) and constant (C) regions which are made up heavy and light chains. The number of domains depends upon the immunoglobulin isotype. Three constant heavy chain regions (CH1, CH2, CH3) domains are noted IgG, IgA, IgD and four constant domains CH1 to CH4 noted on the heavy chains of IgE and IgM. Particular biological properties of immunoglobulins IgG and IgM are especially associated with certain heavy chain domains and complement fixation. The hinge region of immunoglobulin structure exists between the CH1 and CH2 domains of the heavy chain. Minor differences in the hinge regions are used to subtype IgG into four subclasses. In IgG molecules there is a specific constant heavy region (CH2 and CH3) which allow for attachment of Fc receptors of monocytes and macrophages.

IgM is a pentamer with a molecular weight of approximately 900 kDa and consists of five subunits with weight of 180kDa each. Each subunit is linked by J chain, which is a sulfhydryl-rich peptide (15 kDa) and it consists of two heavy chains μ and two light chains of type κ or λ. J chain contribute to integrity and stability of the pentameric structure of IgM. The Fc fragment of IgM is a cyclic pentamer with molecular weight of approximately 340 kDa.

There are two forms of IgA. One is a monomer and other exists in a polymeric form- as dimers or trimers composed of two or three identical monomers respectively and are joined by a J chain. IgA is located in different parts of the immune system depending upon the subclass. In serum IgA is found in both

monomeric and polymeric forms. Secretary IgA is normally found in mucosal tissues of the body. The polymeric form of secretary IgA acquires a glycoprotein secretary component and when it passes through epithelial cell walls of mucosal tissues and this appears in nearly all body fluids. Another importance of the IgA is that it can increase the effect of IgG induced RBC hemolysis.[13]

In Sudipta Sekar Das et al study, Multiple red cell bound antibodies had (83.3%) severe in vivo hemolysis compared to only (22.6%) single autoantibody.[14]

SUBTYPE OF IgG AND HEMOLYSIS

IgG antibodies is subdivided into four subclasses on the basis of minor structural differences in the hinge region of the IgG structure. The IgG subtypes are IgG1, IgG2, IgG3 and IgG4. The ratio of κ to λ in human IgG is 2:1, but the ratio is 1:1 and 1:8 for individuals with IgG2 and IgG4 subclasses respectively. The disulfide bonds linking the heavy chains also act as a factor for structural variations among the different subclasses. While IgG1 and IgG4 have two bonds each, IgG2 and IgG3 have four and five bonds respectively. This disulfide bond is responsible for flexibility to the hinge region of the subclasses of IgG molecule and the distance or angle of Fab fragments determines the antigen it can accommodate.[15]

Each of the subclasses exhibit differences in properties including placental transfer and complement fixation. While IgG1 and IgG3 binds to complement C1q molecule more strongly than IgG2. IgG4 doesn't bind at all and cannot activate complement cascade.[16] In case of IgG2, there are two alleles of the particular gene that encodes the FcRIIa receptors on macrophages. As a result some people have low affinity receptor for IgG2 and these subjects show positive DAT in the presence of IgG2 autoantibody without the signs of hemolysis. Subjects with high affinity

receptors have the potential to destroy IgG2 coated cells. However IgG2 mediated hemolysis also depends upon the antigen specificity.

Macrophages have IgG Fc receptors only for IgG1 and IgG3[17, 18] therefore; the quantity and type of IgG on the red cell surface influence the degree of hemolysis. Studies done on IgG subtypes revealed that red cells coated with IgG1 alone or in combination with IgG2 or IgG4 require an average of 2000 molecules of IgG per red cell to stimulate phagocytosis and rosette formation in vitro. However in case of IgG3 subtype, an average of 230 IgG3 molecules per red cell is required for monocyte binding.[19] Only IgG1 and IgG3 are efficient in activating complement. Destruction of red cells is further enhanced when complement is also present on the red cell membrane.

Since two molecules of IgG in close proximity is required to bind C1q and activate complement system,[20] there must be a sufficient number of antibody molecules and antigenic sites for complement attachment. Once C1 is bound, C4 and C2 activated to form C3 convertase, which then cleaves C3 and C3b. Several hundred molecules of C3b bound to the red cell membrane through the action of a single C3 convertase enzyme complex.[21]

The IgG Fc and complement receptors act together to enhance the binding of red cells coated with IgG and complement. Removal of IgG-coated red cells with or without complement occurs primarily in the spleen. However, spleen plays dominant role in destruction of IgG coated red cells. As there is no C3 or iC3b in normal plasma, there is no competition for the macrophage complement (CR1 and CR3) receptors in the liver; thus the splenic macrophages have no more efficiency than Kupffer cells in destruction of C3-coated RBCs. C3b does not remain on the red cell very long. If the C3b-coated red cell does not interact, or if the interaction with a C3b receptor on a macrophage is inefficient, the cell-bound C3b is denatured.

Naturally occurring complement control enzymes factors H and I cleave C3b molecules, first forming iC3b, then a second cleavage breaks away C3c, leaving only C3dg on the RBC membrane. C3dg can be further cleaved in vitro, using trypsin, to C3d.[18] The spleen acts as a fine filter and the liver acts as a coarse filter of sensitized red cells in immune hemolytic anemias (IHA).

Of the subclasses of IgG, IgG3 has the highest affinity for the FcγR and therefore most efficient causing extravascular hemolysis (IgG3> IgG1> IgG2>>> IgG4).[22, 23]

The most prominent subclass of warm autoantibody found in most warm AIHA patients is IgG1.[26] IgG3 antibody is mostly found in combination with other subclasses in 5% of patients and found as a solitary antibody in 3% of patients.[28] R.J Sokol et al., in their study found that IgG3 is rarely found as a single class and it is found alone in 1% in their study population.[13]

J. Fabijan´ska-Mitek H. Lopien´ska B. Zupan´ska The IgG subclasses were detected by the gel test in 100% of the AIHA cases. In 95.5% of the patients IgG1 was detected, either alone (59.1%) or together with other subclasses (36.4%). Multiple IgG subclasses were also associated with more pronounced haemolysis: severe haemolysis in 61% of the cases, moderate in 33%, most of them with IgG3. In most of the cases with mild haemolysis, only IgG1 was detected (69%); if other subclasses were found, they were IgG2 or IgG4, but never IgG3.[29]

In Sudipta Sekar Das et al study,. IgG1 alone or in combination with IgG3 were the predominant IgG subclasses. In 46.5% of the patients the subclass was IgG1 or IgG3 or both.[14]

Janet M. Pollock, John M. Bowman distribution of anti-D subclasses in HDFN was 3% IgG3 alone, 33% IgGl alone, and 64% IgGl and IgG3.[30] Frankowska

and Gorska found that 87.6% pregnant women contained IgG1 Rh antibodies, 23% contained IgG2 antibodies, 56.9% contained IgG3 antibodies, and 7.7% contained IgG4 antibodies. Most commonly, the sera contained IgG1 alone (33.9%) or IgG1 + IgG3 (32.3%); no sera contained IgG2 and/or IgG4 without IgG1 or IgG3.[31]

In the study conducted by Schanfield, neonates born with RBCs sensitized with IgG1 Rh antibodies had lower mean cord hemoglobin and higher cord bilirubin levels, with a lower postnatal bilirubin rise, than neonates with IgG3-sensitized RBCs. He suggested that this finding related to the preferential placental transfer of IgG1, which allowed for a longer period of in utero IgG1 sensitization. In contrast, neonates with IgG3 sensitization tended to have higher mean cord hemoglobin and lower cord bilirubin levels, but a higher postnatal rise in bilirubin. This was thought to be due to the shorter in utero exposure to IgG3 but the relative higher efficiency of IgG3 in causing postnatal RBC destruction.[32]

Sanford et al., Patients with acute hemolytic transfusion reactions or delayed hemolytic transfusion reactions (DHTRs) may have a positive direct antiglobulin test (DAT) result with immunoglobulin (Ig)G and/or complement fixation (eg, C3d).[33]

IMMUNOGLOBULINS AND COMPLEMENT ACTIVATION

The complement system is a complex group of more than 20 circulating and cell membrane proteins with various functions within the immune response. Primary roles include direct lysis or destruction of cells, bacteria and enveloped viruses as well as helping with opsonisation to facilitate phagocytosis.

The complement proteins are activated by cascade of events mainly through three pathways:

1. Classical
2. Alternative and
3. Lectin pathways.

The three pathways converge at the activation of the component C3. The classical pathway is activated by the binding of an antigen with IgM, IgG1 or IgG3 antibodies. The activation of classical complement pathway is initiated when an antibody binds to antigen. This allows the binding of the complement protein C1 to Fc fragment of IgM, IgG1 or IgG3 antibody. Ability of IgG antibody for complement activation depends on cell surface antigenic concentration and antigen clustering, in addition to antibody avidity and concentration. IgM is large and has Fc monomers close to each other on one immunoglobulin molecule; therefore only one IgM molecule is sufficient to activate complement.

The C1 component is a complex composed of three C1 subunits C1q, C1r and C1s which are stabilized by calcium. In the C1 complex, C1q is responsible for catalysing C1r to generate activated C1s. activated C1s is a serine-type protease. The C1qrs complex acts on C2 and C4 to form C4b2a. C4b2a uses component C3 as a natural substrate and C3b which is formed attaches to microbial surface. Some C3b attaches to C4b2a complex and resulting C4b2a3b complex functions as C5 convertase. The C5 convertase acts on C5 to produce C5a (a strong stimulator of anaphylotoxins) and C5b, which binds to the cell membrane and helps C6, C7, C8 and C9 complements to the cell membrane. When C5b along with C6, C7, C8 and C9 are bound membrane attack complex is formed; this causes cell lysis.

In patients with cold agglutinin disease, serum levels of complement proteins C3 and C4 are low in most patients because of constant consumption, which may limit further extra and intra vascular hemolysis.[34]

Salama and Mueller-Eckhardt observed that the patients with DHTRs found to have IgG and C3d detectable on their RBCs after the reaction.[35] Ness and coworkers detected RBC-bound IgG by the DAT in all (100%) patients; however, 56% of them also had RBC-bound complement.[36]

CAUSES OF POSITIVE DAT

Patients with Clinical and/or laboratory evidence of hemolysis and DAT positivity are broadly classified into Immune hemolytic anemia (IHA). Further, they are classified into AIHA, DiAIHA and Alloimmune Hemolytic Anemia (Transfusion reaction and HDFN).[24]

Autoimmune Hemolytic Anemia

The Classification of AIHA is patho-physiologically based and divides AIHA into warm, mixed or cold-reactive subtypes. This thermal-based classification is based on the optimal autoantibody-RBC reactivity temperatures. AIHA is further subcategorized into idiopathic and secondary with the secondary AIHA being associated with a number of underlying infectious, neoplastic and autoimmune disorders. {Uncompensated autoantibody-mediated red blood cell (RBC) consumption is the hallmark of autoimmune hemolytic anemia (AIHA)}.[2, 13, 37, 38]

Further sub-classification of cold AIHA (cAIHA) includes primary and secondary cold agglutinin syndrome (CAS) and paroxysmal cold AIHA.[37-39] While warm AIHA and cold AIHA constitute much of the AIHA prevalence some less frequent types do arise, namely mixed-type AIHA (mAIHA) and drug-induced AIHA (diAIHA). In most cases AIHA is confirmed by a positive direct antiglobulin test (DAT). diAIHA is even more rare, afflicting an estimated 1 in 1 million. diAIHA can be classified into sub-categories depending on if the drug is required to be present for hemolytic activity (drug-dependent AIHA), or if hemolytic activity is

observed without the drug present (drug-independent AIHA).[40] While each subtype of AIHA is innately part of the same family, pathogenesis, diagnostics, treatments, and prognosis vary greatly. Accurate diagnosis is therefore crucial to assess clinical manifestations, predisposing factors and treatment optimization.

Warm autoimmune hemolytic anemia (WAIHA)

An estimated 1 in 80,000 are afflicted by WAIHA, constituting about 75% of all AIHA cases.[40] Idiopathic/Primary AIHA accounts for approximately half of all WAIHA.[40] Warm AIHA is the subtype that most often affects children age between 2–12 years.[42]Secondary WAIHA is associated with various conditions including infectious mononucleosis, systematic lupus erythematosus (SLE), autoimmune hepatitis, human immunodeficiency virus, and other lymphoproliferative or autoimmune disorders.[13, 35, 39, 43] Of AIHA-associated lymphoproliferative disorders chronic lymphocytic lymphoma (CLL) is the most common cause.[44] In fact, roughly 11% of CLL patients develop secondary WAIHA, while an annual incidence of 2–3% is observed in patients with non-Hodgkin's and Hodgkin's lymphoma.[42-44] The polyclonal immunoglobulin (Ig) class IgG is typically involved in the autoantibody activity of warm AIHA (WAIHA), showing maximal reactivity with erythrocytes at 37 °C. Less frequently, WAIHA can be associated with IgA and IgM. WAIHA exhibits a depleted immune tolerance of RBCs commonly due to the binding of self-antibodies to Rh proteins, causing Fc-gamma receptors to mediate removal of RBCs extravascularly within the spleen.[37] WAIHA has recently been linked to a number of immune system imbalances. Interleukin-12 (IL-12) and interleukin-10 (IL-10) imbalances are believed to mediate the altered immune response in some patients with AIHA.[44,47] The pattern of IL-10 and IL-12 production is generally thought to play a role in the pathogenesis of WAIHA and correlates with increased activity of the Type-2 Helper T Cell (Th2)

pathway and the inhibition of Type-1 Helper T Cell (Th1) pathway.[46,47] The domination of the Th2 pathway leads to increased autoantibody production mediating AIHA.[46-50]

Most observers have reported a somewhat higher incidence in females than in males. In idiopathic cases of WAIHA, Allgood and Chaplin reported that 60% of patients were female;[51] Dausset and Colombani reported that 61% of patients were female;[52] Pirofsky indicates that 64% of patients were female; [41] Dacie reported 58% of patients were female; [39] Dacie and Worlledge reported that 59% of patients were female;[53] and, in the series reported by Böttiger and Westerholm, women predominated in all age groups with the exception of the youngest (0 to 14 years), in which the sex distribution was even.[54] In secondary WAIHA, the percentages are more varied, perhaps depending on the incidence of underlying diseases seen in referral centers.

Cold autoimmune hemolytic anemia (cAIHA)

Cold agglutinin syndrome (CAS)

CAS is much less prevalent than WAIHA, comprising about 15% of all AIHA cases, primarily occurring in the middle aged or elderly. CAS causes AIHA in a complement-dependent manner where autoantibody-dependent lysis is mediated primarily by C3 proteins, leading to intravascular hemolysis upon detachment of antibodies at 37°C.[55] Targeted RBC phagocytosis is primarily mediated by liver Kupffer cells while the membrane attack complex (MAC) is a minor mechanism if the IgM titre is relatively low. The presence of cold stress increases autoantibody activity, facilitating RBC lysis particularly in the extremities. A notable feature of CAS is a high variability in hemolysis, and in turn the need for transfusions varies greatly from patient to patient.[56] The degree of hemolysis in CAS patients is

primarily dependent on active autoantibody concentration, rather than the more abundant membrane bound C3 protein concentration.[56, 57]

Paroxysmal cold hemoglobinuria (PCH)

PCH, also known as Donath–Landsteiner syndrome and is a form of cAIHA activated primarily by polyclonal IgG antibodies (Donath–Landsteiner antibody). Similarly to CAS, PCH activates complement at cold temperatures. Complement activation is via P-antigen binding on RBCs with subsequent intravascular hemolysis being initiated upon rewarming to normal body temperatures. PCH is considered a form of secondary AIHA and typically develops within the first week after infection most often seen in children. The infections are primarily upper respiratory, and the causative agent is often not identified. Late-stage or congenital syphilis was historically linked to cases of PCH in adulthood but this is becoming less and less common.[57]

Mixed-type AIHA (mAIHA)

mAIHA is characterized by the presence of both warm and cold type antibodies as well as both IgG and IgM antibody subtypes. mAIHA accounts for less than 5% of the total AIHA incidence, and is even less common in children.[58] mAIHA can be both idiopathic or arise secondarily from malignant or autoimmune disorders such as SLE or lymphoma. It can be difficult to determine which autoantibodies (IgG or IgM) and the required thermal range are causative. Patients with mAIHA can have both warm and cold components that can react with different antigens.

DRUG-INDUCED IMMUNE HEMOLYTIC ANEMIA (DIIHA)

DIIHA is relatively rare, may go undiagnosed in many cases, and the magnitude of hemolysis can vary widely. There are an estimated 150 drugs known to be associated with DIIHA and are categorized by drug-independent (via auto-antibodies) and drug-dependent antibodies.[59-62] Drug-dependent AIHA can be categorized into two subtypes:

1) Hapten type which is due to the noncovalent binding of the drug to the RBC which is then targeted by the autoantibody in a drug-dependent manner:

2) drug-autoantibody immune (ternary) complexes that are mediated by a complement-dependent hemolysis that is drug dependent.

Drug-dependent antibody (DDAB) activates a response only while the drug is present. This class is the most common case of diAIHA and can be mechanistically variable depending on the molecular nature of the drug and its RBC interaction. DDAB may specifically attach to the drug, the drug's metabolites, and/or drug-RBC neoantigens. Antibiotics cefotetan and high doses of penicillin are the best understood mediators of diAIHA. The binding of the DDAB mediates RBC phagocytosis via Fc receptor-mediated mechanisms similar to WAIHA with the notable difference that the autoantibody binds directly to the RBC in WAIHA and to the drug-bound RBC in diAIHA.

Other drugs such as ceftriaxone and piperacillin interact with the membrane of RBCs but bind via RBC neoantigens. It is uncertain how drug binds to the membrane and if the complex formed is covalent or loosely bound. The drug and RBC membrane form an immune complex mediating DDAB binding to the drug, membrane, or equal proportions of the drug–membrane complex.[62]

In contrast drug independent antibodies (DIABs), are capable of creating an autoimmune response in the absence of the offending drug. Various mechanisms exist by which drugs (i.e. fludarabine, cladribine, and methyldopa) stimulate autoantibody formation via adsorption, immune dysregulation, or other mechanisms none of which have been fully elucidated. The most common drug usage linked to diAIHA is methyldopa, which can continue in a subject months after cessation of the drug. The common treatment practice for diAIHA is mediated via blood transfusion and discontinuation of the offending agent. Most drugs are cleared from the system quickly and the drug-dependent antibodies only persist in the case where there is a persistence of RBC membrane-bound drugs.[61-62]

ALLOIMMUNE HEMOLYTIC ANEMIA

Hemolytic Diseases of Fetus and New-born (HDFN)

The first antibody to be described as a cause of HDFN was anti-D. A positive DAT in a new-born result is due to transplacental transfer of IgG antibodies, which are present in maternal serum and directed against antigens on fetal and neonatal red blood cells (RBCs). Such antibodies may cause destruction of neonates' RBCs and shorten their life span, leading to clinical manifestations of HDN and various degrees of hyperbilirubinemia and anemia.[63] The factors that can lead to a positive DAT in neonates are mainly the ABO incompatibility between the new-born and the mother, maternal alloimmunization, and very seldom maternal autoimmune hemolytic anemia.[1] ABO incompatibility with a positive DAT is considered a major risk factor for the development of severe hyperbilirubinemia and neurotoxicity.[64, 65] By contrast, some studies report that the positive DAT has only a poor predictive value for severe hyperbilirubinemia.[68,69]

Hemolytic Transfusion Reactions (HTR)

The most severe alloimmune hemolysis is an acute transfusion reaction caused by ABO-incompatible red blood cells. For example, transfusion of A red cells into an O recipient (who has circulating anti-A IgM antibodies) leads to complement fixation and a brisk intravascular hemolysis. Within minutes, the patient may develop fever, chills, dyspnea, hypotension, and shock. Delayed hemolytic transfusion reactions occur three to 10 days after a transfusion and usually are caused by low titer antibodies to minor red blood cell antigens. On exposure to antigenic blood cells, these antibodies are generated rapidly and cause an extravascular hemolysis. Compared with the acute transfusion reaction, the onset and progression are more gradual.[70]

CLINICAL FEATURES

WAIHA

Clinical picture of WAIHA is highly variable. Most patients have symptoms correlated to anaemia, such as fatigue, palpitations and shortness of breath. Occasionally massive haemolysis manifested by haemoglobinuria, haemglobinemia and profound anaemia can be seen with secondary WAIHA.[72] Naithani et al found that most common symptoms in their study were related to anaemia.[27] The associated illness often dominates the clinical picture. AIHA may proceed by months or even years before the development of diseases such as SLE. Physical findings in idiopathic AIHA were pallor, resting tachycardia mild jaundice; fever may be present. The spleen is only moderately enlarged.[74] Pallor was universal finding in 98% of patients.[2]

Evidence of hemolysis was established with peripheral blood spherocytosis and fragmented cell, with elevated reticulocyte count, erythroid hyperplasia in the

marrow, unconjugated hyperbilirubinemia, and a positive direct antiglobulin test (DAT) with or without indirect antiglobulin test (IAT).

COLD AIHA

They often present with symptoms of chronic anaemia. Episodes of acute hemolysis occur after cold exposure, which is presented as haemoglobinuria, haemglobinemia.[73] The clinical course of Cold AIHA is characterised by either chronic anaemia or episodes of haemolysis, depending upon the thermal amplitude of the cold agglutinin.[23] In children, PCH appears 1 to 2 weeks after an episode of upper respiratory tract infection. Usually, the onset of haemolysis is signalled by a recurrence of fever and then the passage of reddish brown urine.[2]

Evaluation for DIIHA should be assessed by a careful history of drug exposure in every patient with AIHA and/or a positive DAT. In general patients with hapten/drug absorption (i.e. penicillin) and autoimmune (i.e. methyldopa) types of diAIHA exhibit mild to moderate hemolysis, with insidious onset over a period of days to weeks. In contrast, the immune or ternary complex-mediated DIIHA (i.e. cephalosporins or quinidine) typically is associated with a sudden onset of severe hemolysis and hemoglobinuria.[60-62]

The diagnosis of anaemia on the basis of physical signs is remarkably difficult and the classic observation of pallor is quite unreliable. Jaundice is most common presenting sign in 39% of the patients in pirofsky's series.[41] Allgood and Chaplin reported that Splenomegaly was present in 57% of the patients with idiopathic AIHA.[51]

HDFN

Features of hemolysis are in the form of anaemia (cord HGB3.5-8g/dl), Jaundice and hepatosplenomegaly along with positive DCT.[63]

THE ANTIGLOBULIN TEST

Red cell coated autoantibodies and alloantibodies are gamma globulins, usually IgM, IgG, IgA, or complements such as C3d, C3c. If they are IgM, they usually directly agglutinate saline-suspended RBCs. In contrast, IgG antibodies often do not agglutinate RBCs but react with the corresponding antigens on the RBC membrane, giving a "sensitized" RBC. Thus, chemically speaking, the RBCs are sensitized with gamma globulin. The AHG will combine with it, crosslinking the sensitized RBCs and causing agglutination. Anti-IgG reacts mainly with the Fc portion (i.e., heavy chains) of human IgG molecules present on the RBCs. The heavy chains are specific for each class Antibodies, however antibodies to light chains may be present in polyclonal AHG as well; in a polyspecific AHG, this has no disadvantages and theoretically may be an advantage by forming extra "bridges" across adjacent light chains and the polyspecific AHG can pick up other immunoglobulins such as IgM, and IgA;.

In 1946, Boorman and coworkers[73] and Loutit and Mollison[74] reported that the RBCs from patients with idiopathic acquired hemolytic anemia reacted with AHG. The first application of the test (i.e., detection of Rh antibodies in serum) became known as the indirect antiglobulin test (IAT),[1] and the second application (i.e., detection of in vivo sensitization) became known as the DAT.[75,76] However, IgM sensitization of RBCs is difficult to detect with the AGT;[72,73] furthermore, IgM antibodies that cause immune hemolytic anemia characteristically, if not invariably, fix complement, which is much more readily detected.[74] IgA antibodies only

infrequently play a role in RBC sensitization, and in such cases, other immune globulin and/or complement components are almost always, although not invariably, found on the RBC surface as well.[81-82]

DAT with a polyspecific antiglobulin serum, which is defined as one that must contain anti-IgG and anti-C3d and may contain antibodies to other complement components (e.g., C3b, C3c, and C4) and to other immunoglobulins (e.g., IgA and IgM). A positive result in a patient with acquired hemolytic anemia generally indicates that the patient's RBCs are coated with IgG, C3dg, or both. Using monospecific anti-IgG and anti-C3, it is then a simple matter to determine which of these two proteins are coating the patient's RBCs. DAT with an antiglobulin serum that does not contain anti-C3d will frequently result in misleadingly negative results in patients with IHAs. This is true in all patients with CAS, 13% of patients with WAIHA, essentially all patients with PCH, and many instances of drug-induced IHAs.

The direct antiglobulin test (DAT) should be performed in every patient in whom the presence of hemolysis has been established. Although some exceptions to this rule might be considered, as when the diagnosis of a congenital hemolytic anemia is evident, the DAT is a simple, quick, inexpensive test that yields useful information. A positive result on a DAT in a patient with hemolytic anemia does, of course, indicate that the most likely diagnosis is one of the immune hemolytic anemias. The predictive value of positive DAT is 83% in a patient with immune hemolysis, but only 1.4% in patients without immune hemolytic anemia.[3]

Original DAT method by CTT able to detect a lower limit of 150 to 200 IgG molecules per RBC[72] and 400 to 1100 C3d molecules per RBC.[4] with introduction of GT in 1990 by Lapiere et al, the method gained importance in laboratory practice of immunohaematology in developing countries including India.[76] Gel cards for

detailed serological characterization of auto and alloantibodies are available. The GT had been reported to be more precise and sensitive than widely practiced CTT.[79,80] A small number of studies concluded that GT showed lower sensitivity mainly in detection of C3d coated red cells.

Severity of haemolysis was correlated with the number of antibodies bound to the RBC and the strength of DAT.[80,81] A positive DAT did not always mean decreased RBC survival. Many studies found out the relationship between the presence or absence of hemolysis and the DAT strength was highly statistically significant.[77,78] S S Das et al, from India also observed a significant correlation between strength of DAT and severity of hemolysis. The predictive value of a positive DAT was 83% in the patients with IHA, but only 1.4% in the patients without IHA. [14] Dorothy Dinesh in her study indicates that the sensitivity of a positive DAT for clinically significant HDN is 85%.[9] Huub H.vanRossum et al estimated the positive predictive value (PPV) calculated was 10% for DAT and eluate.[83] It should be emphasized that determination of the presence or absence of hemolysis, should logically precede the performance of the DAT.

Therefore, interpretation of DAT must be done along with clinical history and other laboratory findings. Further evaluation of a positive DAT in a patient with clinical and laboratory evidence of haemolysis includes testing for clinically significant antibodies to RBC antigens and testing an eluate.[61] A significant correlation between small increases of cell bound immunoglobulins and haemolysis was shown in 25% of patients with evidence for immune mediated haemolysis.

ELUTION

Discovery of Elution Technique

The first antibodies coating RBCs elution technique for cold antibodies is the heat elution discovered by Landsteiner and Miller as early as 1902. Landsteiner was also instrumental in developing the second technique for eluting antibodies from RBCs. In co-operation with van der Scheer, he created a method for dissociating azostromato-antibody complexes. This method was modified by Kidd, he eluted the antibodies by exposing stroma to citrate buffer having a pH of 3.2 to 3.4 at room temperature.[84, 85]The freeze-thaw is Weiner's method that destroys RBCs, 50% cold ethanol used for precipitating the stroma and recovering antibody from the precipitate stroma with saline 37°C.[83] For testing IgM antibodies eluates, hemoglobin-free elute are important but they are not essential for indirect antiglobulin tests (IAT).[84] For this objective, Harry Rubin created the ether elution method at 37°C for warm antibodies; in addition some observations were made on the sensitized red blood cells of patients with autoimmune hemolytic anemia.[86] Rubin's ether elution method is dangerous for the blood bank worker. Ether is highly flammable and must be strictly regulated in regard to its use and storage.[87] Rekvig and Hannestad and Bush created glycine-HCl elution method for use with intact RBCs instead of stroma.[88] And these two creations have been developed to modern commercial elution kits such as Elu- Kit II, Gamma Biological Inc. Houston, TX and DiaCidel Elution Kit, DiaMed AG, Switzerland.[89] Another elution method was described by Chan-Shu and Blair, by Bueno R. al using xylene that elution technique was superior to methods using ether, digitonin-acid and heat.[90]

In recent years, various methods of antibody dissociation have been developed that do not destroy the RBCs. The objective is to remove either IgM or IgG autoantibody in a way that permits accurate phenotyping of the RBCs. Three

methods have been developed to permit phenotyping of IgG-coated RBCs with reagent antisera require using by the Indirect Anti human globulin test. The first, Edward et al have Investigated the quinolone derivative chloroquine diphosphate (200 mg/ml, pH 5.0) to dissociate antibodies without denatured red cell antigens. They found the chloroquine dissociation technique to be of value in the examination of red blood cells with a positive DAT, either or the qualitative or quantitative expression of antigen.[91] The second, other investigators studied the effect of acidic ethylene diamine tetra acetic acid (EDTA)-glycine mixtures to remove IgG from RBCs without destroying RBC antigens. The third, Caruccuo L. et al found that the formamide method was efficient in removing antibodies from RBCs. The patient samples with a positive DAT had antibodies recovered with the same specificity when compared to the acid-based technique.[85] The preparation time length was similar for both formamide and acid based methods.[85, 86, 88]

Approximately 80% of the patients with AIHA have autoantibodies in their serum as well as on their RBCs.[9] The antibodies in the serum or plasma and the antibodies eluted from RBCs were detected by IAT(i.e antibody screening).[93] Many panagglutinins were believed to react with a basic determinant of the Rh antigen system, as they fail to react with Rh null RBCs which has no Rh antigens on them.[93] Warm autoantibodies are mostly panagglutinins, reacting with every cell in the diagnostic RBC panel.

Elutions were not typically performed on DAT positive only for complement, as these molecules would not be expected to have antigen binding specificity. However, elution tends to produce a more concentrated antibody solution, hence the reactions are stronger.[11] Autoadsorption and antigenic phenotyping could help in differentiating autoantibodies and alloantibodies, especially if the patient had not been transfused recently.[94] Wikman A et al had 28%

of alloantibodies in AIHA patients all of them fell in moderate hemolysis group.[77] Similarly the study conducted by Branch DR and Petz LD 25-47% of sera from AIHA patients showed the presence of alloantibodies.[139]

Autoadsorption could also be used to cross match donor RBC units or patients with warm autoantibodies (only for the patients who have not been transfused recently).[11]

FACTORS THAT INFLUENCE THE OUTCOME OF ELUTION

1. Incomplete washing
2. Dissociation of antibodies prior to elution
3. Storage changes to organic solvents.
4. False-positive eluates with acid elution kits.
5. Antibody binding to glass surfaces.

APPLICATIONS OF ELUTION

1. In the initial evaluation of samples suspected to contain warm autoantibodies. To report the antibodies as "auto," one must demonstrate either that the antibody can be eluted from the patient's RBCs or that they are adsorbed by those RBCs. Once autoantibodies have been demonstrated by elution, there is no need to repeat the elution study each time a sample is submitted for DAT.

2. In the evaluation of a positive DAT/autocontrol that is performed as part of an antibody identification study. Even here, elution studies should be restricted to instances in which the patient has been recently transfused and serum studies are inconclusive. Rarely will elution studies reveal an alloantibody that is not readily detectable in the serum.

3. When there are clinical signs and symptoms of immune hemolysis. Elution studies can be informative even when the DAT is negative, as in "Coombs-negative" autoimmune haemolytic anemia, or sometimes in the investigation of a delayed hemolytic transfusion reaction.

4. In the evaluation of suspected haemolytic disease of new born (HDN), when screening tests for unexpected antibodies on maternal serum are nonreactive, and paternal RBCs are ABO incompatible with maternal serum. Testing an eluate prepared from the infant's cells against the paternal RBCs may indicate a maternally derived alloantibody to a low-prevalence paternal antigen. Blood also may be cross matched against an eluate when the infant needs an exchange transfusion and maternal serum is unavailable.

5. Phenotyping red cells in patients with a positive DAT.

TYPES OF ELUTION METHODS

Heat

An increase in temperature results in displacement of the equation to the left, dissociation of [AgAb] at the molecular level, heat increases the thermal motion of atoms and molecules, leading to dissociation.

An increase in heat also causes conformational changes to proteins, leading to loss of structural complementarity. At 56˚C denaturation of RBC membranes occurs, as evidenced by the haemoglobin stained eluates prepared by the method of Landsteiner and Miller. Calvin et al., and others have shown that RBCs incubated at 56˚C for 5 minutes lose their Rh antigens. Incubation of RBCs at 56˚C for 10 minutes destroys Fya and Jkb antigens, and weakens the expression of M and P1

antigens. Membranes isolated from the treated RBCs give an abnormal electrophoretic pattern when subjected to polyacrylamide gel electrophoresis.

Freeze-Thaw

Extracellular ice crystals that form as RBCs freeze attract water from their surroundings. This increases the osmolarity of the remaining extracellular fluid, which then extracts water from the RBCs. The ice crystals cause the RBCs to shrink in size and also mechanical damage to RBC membranes which results in cell lysis. Some dissociation of antibody may occur because of changes in the ionic strength of the extracellular fluid and rearrangement of water molecules at the cell surface. Disruption of RBC membranes undoubtedly leads to denaturation of antigens, some of which may be shed from rigid cell membranes; such rigidity can be presumed to exist with RBCs at sub-zero temperatures.

The only tenable explanation for the mechanism of antibody elution by the Lui freeze-thaw method is one of conformational changes to membrane structures resulting from the dramatic and rapid changes in temperature that is inherent in this technique.

Sonication

High-frequency sound waves cause a rapid alternation in pressure within liquids, causing formation of minute gas bubbles. When they reach a critical size, they implode. The produced shockwaves exerts considerable shearing forces. Antibody molecules are shaken off the RBCs. The considerable amount of heat produced during sonication, resulting from the implosion of the gas bubbles, will also contribute to antibody dissociation.

pH Changes

Proteins become either negatively charged at a high pH (anionic state) or positively charged at a low pH (cationic state). In either state, proteins lose their ability to attract one another through electrostatic bonding and may actually be forced apart by repulsion of like charges. H + ions and OH- groups,which abound in low- and high-pH solutions, respectively, are attracted to opposite charges on polar amino acids such as lysine, arginine, and histidine (positively charged R groups), and aspartic and glutamic acids (negatively charged R groups). This causes changes to the tertiary structure (molecular unfolding) of proteins, and at extreme alkaline pH levels, the secondary structure of proteins may be affected by disruption of their peptide bonds.

Chaotropic Ions

Cl-, I-, and SCN- ions literally cause chaos to proteins. They bind to charged groups on amino acids that govern the tertiary structure of proteins. Thus, molecular unfolding and even disruption of peptide bonds can occur when proteins are suspended in solutions of salts that include these ions. At high salt concentrations, the "salting-out" of proteins occurs, which involves inward folding of polypeptides and leads to a decrease in protein solubility. Thus, Chaotropic ions cause considerable conformational changes to proteins. With respect to the effect of chaotropic ions on the forces involved in the first stage of hemagglutination reactions, electrostatic shielding of charged groups by counterions leads to weakening of the forces of attraction between antigen and antibody. Also, because solutions containing high concentrations of salts can attract water from a place of lower salt concentration, there may be some rearrange merit to the ordered water molecules at the RBC surface. This will undoubtedly influence the hydrophobic effect involving van der Waals interactions.

Organic Solvents

Organic solvents, such as ether, denature or destroy antigens, whereas antibody molecules are not affected. This occurs by dissolution of the RBC membrane bilipid layer. They demonstrated that the forces of attraction arising from van der Waals interactions can be changed to forces of repulsion when the..J surface tension of the liquid medium stir rounding the RBCs is lowered to a point between the surface tension of the antigen and its binding site on an antibody molecule. Although performed the tests using DMSO, other organic solvents (ether, xylene, chloroform, etc.) likely exert a similar effect.

Chloroquine Diphosphate

RBCs suspended in chloroquine diphosphate have a reduced electrophoretic mobility. Antigens mad antibodies are not denatured, and normal electrophoretic mobility is restored after chloroquine removal. Thus, it appears that the effect of chloroquine may be one of neutralization of charges by counterions, similar to but milder than the effect of chaotropic ions.

Thiol Reagents

It is well known that the sulphydryl compounds DTT and 2-ME disrupt interchain disulfide bonds of pentameric IgM. The spontaneous agglutination of RBCs coated with cold autoantibodies is abolished simply because the IgM molecules on the RBCs fall apart. Similarly, IgG warm autoantibodies can be removed from RBCs by adding either ficin or papain to DTT (ie, ZZAP reagent). These enzymes cleave IgG molecules at a protease sensitive site just below the hinge-region, thereby exposing the intrachain disulphide bonds to the action of DTT.

Ole Petter Rekvig and Kristian Hannestad in their study Acid elution of blood group antibodies from intact erythrocytes describes that Elution of antibodies from intact human and sheep erythrocytes at pH 3.0 than heat elution and ether elution. They concluded that acid elution at pH 3.0 gave the highest yield out of the three methods.[88]

Rahul Katharia and Rajendra K. Chaudhary in their study demonstrated that Heat elution was equally potent as Glycine-HCl/EDTA in removing antibodies from in vitro sensitized red cells, decrease in DAT positivity was not as effective on in vivo-sensitized red cells and red cells coated with autoantibodies. Chloroquine di phosphate was effective in removing antibodies attached from sensitized intact red cells. It is not as potent as Glycine-HCl/EDTA and heat elution. They concluded that Glycine-HCl/EDTA elution method was more effective in reducing strength of reaction in both in vivo and in vitro sensitization.[8]

Huub H.vanRossum et al., study explains the high sensitivity of both techniques (DAT and Elution) on detecting neonatal erythrocytes sensitized with anti-A and anti-B. Especially for A/O-incompatible pregnancies it appears that some degree of sensitization of neonatal erythrocytes with maternal IgG anti-A occurs regularly. “sub clinical” erythrocyte sensitization results in a low positive predictive value and specificity for both DAT as well as eluate screening. Screening for HDN by DAT results in many false positive results. In cases of ABO-incompatible pregnancies large percentages of positive neonatal DAT results are observed in the absence of clinical jaundice.[83]

Elie Richa et al found that Micro+ DATs yielded a lower rate of new antibody detection (5.5%) than the combined groups of macroscopically positive DATs, 12.2% ($p = 0.047$). They concluded that eluate testing in the setting of micro+ DATs should not be a standard practice.[95]

R.H. Finck et al., performed elution with Glycine acid/Elu-kit II in DAT positive cord samples. Antibodies were eluted from all DAT positive cord blood RBCs (2 Jk^a and 5 ABO HDFN). No antibodies were detected in the last wash fluids by either method for all cord blood samples included in the study. They concluded that apart from ABO HDFN all other antibody mediated HDFN can be diagnosed antenatally. However, Elution can be of useful value in diagnosing clinically significant ABO HDFN and difficulty in obtaining maternal serum for other HDFN.[96]

Marilyn Johnston FM, Mary Kay Belota, observed that 68% of patients, in which 37% yielded positive eluates and 63% had nonreactive ones. Of the positive elution studies, 73% demonstrated only warm autoantibody on red blood cells. 2.5% of these had warm autoantibody in serum as well. 3% of these specimens were from previously transfused patients and had alloantibody/ies in serum. They concluded that positive DAT investigation and elution studies appear to be clinically helpful in investigating delayed hemolytic transfusion reactions and identifying implicated alloantibodies.[97]

SPECIFICITIES OF ANTIBODIES IN ELUATE

Wiener and coworkers suggested that 37°C-reactive ("warm") autoantibodies might be directed against the "nucleus of the Rh-Hr substance".[98] Pirofsky and Pratt, in 1966, compared the reactions of alloanti-Rh and "warm" autoantibodies with RBCs from a large variety of primates and non-primates and essentially agreed with the findings of Wiener and coworkers.[99]

Autoanti-e was the most common reported specificity; it has been pointed out that the reported relative incidence of different specific Rh autoantibodies

corresponds well with the incidence of Rh antigens in the population (i.e., e is present on the RBCs of approximately 98% of the population).[39]

COLD AIHA

The main group of antigen recognized by human cold agglutinins (Cold AIHA) have been defined on a serological and biochemical basis. It is mainly the Ii antigens. They are protease- and sialidase-resistant differentiation antigens. I antigen is fully expressed on adult and i antigen is fully expressed on fetal RBCs. Anti-i recognizes linear poly- *N*-acetyl lactosamine or type 2 chains, which are converted into I antigens in the first year after birth by branching. Jenkins and coworkers found that sera containing cold autoagglutinins that had previously been called "nonspecific cold agglutinins" had anti-I specificity and it is more common to other cold autoantibodies.[100]

HEMOLYTIC TRANSFUSION REACTIONS (HTR)

Based on the study done by Grove and Rasmussen the most common antibodies to cause HTR were anti-K, anti-E. The next most common antibodies were anti-Fya, anti-c, and anti-Jka.[101] The Mayo Clinic's 10-year study also found anti-K to be the most common cause of HTR. They found that second common antibody was anti-Jka and anti-Fya was equal to anti-E as the third most common offenders. [102-105]

When DHTRs were examined separately, anti-Jka and anti-E were found to be the cause of HTRs more often than anti-K. Anti-Jka and anti-E were followed by anti-K and anti-Fya, respectively, as causes of DHTR.

HDFN

Historically, the next most common antibody after anti-D was anti-E, followed by anti-c, -Jka, and -K, followed by anti-C, -s, -e, -cE, -Fya.[106]

In 1964, Giblett reported that 93% of antibodies detected in sera of pregnant women were anti-D (or anti-C+D).[107] Kornstad et al., reported that in Norway, the occurrence of new cases of anti-D had fallen from 0.6% to 0.2%.[108]

Mollison et al., The commonest IgG red cell antibodies in human serum are anti-A and anti-B, although, relatively high concentrations are found only in group O subjects. Although ABO haemolytic disease is common, relatively few infants are severely affected; the proportion is higher in some populations than in others. Haemolytic disease due to anti-D tends to be more severe than haemolytic disease due to anti-c. As a cause of death from haemolytic disease, anti-K is next in importance after anti-c.[109]

TITRATION

After test findings suggest CAD, the antibody titer and thermal activity should be determined. The latter is essential to prevent over-diagnosis, because most agglutinins are clinically insignificant. [110] G. Garratty, L. D. Petz and J. K. Hoops IN THEIR study of CAS, the cold agglutinin titres (CAT) at 2°C were found to range from 1024 to 12 000, the upper thermal limit for agglutination in vitro being 25-37˚C. Most of the sera had titres between 2000 and 64000 and reacted up to 28-32°C. It should be noted that all these tests were with saline suspended red cells.[111] According to Pirofsky (1969), 'The diagnosis of cold hemagglutinin disease is dependent on demonstrating a cold acting erythrocyte autoantibody that differs in two fundamental aspects from those seen in normal serum. Titres are generally elevated over 500 at 0˚C; in normal serum, the cold hemagglutinin titre rarely

exceeds 64. In general a high thermal reactivity of the pathologic cold acting erythrocyte autoantibody is also observed.[99]

One study showed only 8% of patients with cold agglutinins displaying clinically significant activity.[112] The titer level is less concordant with disease activity because hemolysis occurs with levels as low as <1:32[113] however, most consider titer levels greater than 1:512 as clinically significant.[112] Berentsen et al reported a median titer of 1:2048,[114] whereas Stone and colleagues observed titers from 1:512 to 1:65 536.[111] After CAD is established, patients should be evaluated for infections, underlying malignancies, and autoimmune disease because more than 70% of cases may be attributable to these processes.[111,113]

Hopkins C and Walters TK in their study says that the thermal amplitude test is performed to determine the reactivity of a cold autoantibody at varying temperatures: 4° C, 22° C, 30° C, and 37° C. Cold autoantibodies that are reactive at temperatures greater than 30° C have the potential to be clinically significant regardless of the antibody titer. Cold antibodies that are reactive at temperatures less than 30° C are not considered to be clinically significant.[115]

MANAGEMENT OF AIHA

Patients with mild and compensated hemolysis did not require any treatment.[70] Patients with AIHA, who presents with severe anemia are in need of blood transfusion. [2] A diagnosis of AIHA is often made in blood transfusion services when autoantibodies are detected during the compatibility testing. Transfusion of patients with AIHA presents with unique set of problems.[116] The indications for transfusion must be considered in light of following facts:

- The risks of transfusion are somewhat increased due to the difficulty of compatibility testing.

- The patient's autoantibody can be expected to cause a shortened life span of transfused red blood cells (RBCs).[117]

Nevertheless, blood should never be denied to a patient with a justifiable need even though the compatibility test might be strongly positive.[117-119] A major difference between transfusing patients with AIHA and those without RBC autoantibodies is that the clinician must consider the time that is required by the transfusion services to do complex serological tests to assure that optimal red cell product is obtained.[2] The clinician and the transfusion service must be in contact with one another so that the transfusion service understands the urgency of the situation and the clinician understands the complexity of the serological studies to be undertaken.[120] The serological investigations are usually time consuming, notifying the need for transfusion and initiation of AIHA workup as early as transfusion considered is essential for the successful management of the severely anaemic AIHA patients.[121] Finally, it should be emphasized that the decision to transfuse must not depend on the serological findings but rather it should be on the patient's clinical status.[2] One of the common mistakes in the management of AIHA is the reluctance to transfuse even those with severe anemia. Such cases had been reported by conley and associates.[122,123] In patients with AIHA and moderate anaemia, the physician should note whether the patient appears acutely ill suggesting of acute hemolysis or compensated gradual loss of RBCs.[124] 50% of patients with WAIHA will respond to adequate doses of corticosteroids during the first week of therapy. Acute PCH seldom lasts longer than 7 to 10 days.[125] The hazards and inconvenience of long term transfusions must be considered. It is preferable to live with mild symptoms of anaemia rather to face the hazards of long-term transfusion therapy.[126]

The RBC autoantibody might react strongly in vitro (eg., 2+ to 4+ in IAT) with all available donor units, thus making the selection of compatible blood for

transfusion impossible. Nevertheless, acute transfusion reactions occur only infrequently. In very rare instances, they develop significant complications such as post transfusion haemoglobinuria, acute renal failure[38, 127] or disseminated intra vascular coagulation.[128, 129]

The term least incompatible is not an official term in transfusion medicine and is not defined in medical literature.[130] It appears to mean that the selection of a unit of blood which gives weaker reactions in the compatibility testing than other incompatible units. This implies, one may perform cross matching using a number of ABO and Rh matched donor units with the patient and finally select the unit that reacts least strongly. The rationale for using "least incompatible" units appears to be that the stronger reactions could be caused by an alloantibody.[118]

Although the donor blood appears to be compatible, the transfused RBCs cannot be expected to survive normally. Mollison has stated that in all those conditions in which a hemolytic anemia was due to some extrinsic mechanism rather than any intrinsic defect in the RBCs, transfused normal RBCs were expected to undergo accelerated destruction.[109] As pointed out by Leger and Garratty, patients who had autoantibodies should receive the same protection from haemolytic transfusion reaction reactions as other patients.[131] American Association Blood Bank's standards indicates that if a patient had received a transfusion or been pregnant within the preceding 3 months, then the sample must be obtained from the patient within 3 days of scheduled transfusion.[132] some investigators suggest that the transfusion of RBCs that are prophylactically antigen matched with the patient's, rather than performing adsorption studies to detect alloantibodies.[133] Providing phenotypically matched blood can provide significant measure of safety.[134]

The one aspect of therapy about which there is a uniform agreement is that corticosteroids should be an initial therapeutic tool in treating patients with AIHA of

warm antibody type.[2] Data in the older medical literature indicates that the initial response was often excellent, with about 80% of patients experiencing prompt reduction in RBC destruction.[40,48,134-136] The onset of the response was usually rapid. Significant haematological improvement is often evident within a few days and most patients demonstrate benefit within 10 to 14 days.[137,138]Although one might be tempted to discontinue steroids more rapidly, recurrences seem to be more likely unless the patients were treated for a minimum of 3 to 4 months with low doses of corticosteroids following subsidence of an acute episode of hemolysis.[139,140] splenectomy had a distinct advantage over other therapeutic options in that it offers the potential for complete and long term remission. The available data suggests that the splenectomy is helpful in complete and long term remission of 50% of WAIHA patients.[137] Transfusions are usually transient benefit but might be required initially because of severity of anemia until the effect of other treatments takes place.[2] In severe hemolysis with significant anemia treatment with corticosteroids was initiated.[72] When anaemia is severe in spite of steroid therapy IvIg might be tried. Azathioprin and Rituximab had been useful in chronic refractory cases.[116]

Childhood AIHA up to 77% of cases are self-limiting, requiring only short term therapy. First line of therapy is corticosteroids, prednisolone starting dose of 2mg/kg/day with response rate of 81% to 100% of children with primary and secondary AIHA. Children compensate better for falling Hb. Hence, transfusion support is usually avoided until there are signs of cardiac compensation. Tests for additional immunological diseases such as autoimmune lymphoproliferative syndrome and a primary immunodeficiency should be done before starting treatment.

MATERIALS AND METHODS

STUDY DESIGN

This cross sectional study was carried out in the Department of Transfusion Medicine and Immunohematology, The TN Dr.M.G.R Medical University, Chennai and Department of Hematology, Madras Medical College, Chennai.

STUDY POPULATION

In this study, patients with clinical and laboratory evidence of hemolysis with Direct Antiglobulin Test Positive Results were included.

STUDY PERIOD

This study was done over a period of one year spanning from August 2017 to September 2018.

SAMPLE SIZE

All DAT positive Immune hemolytic anemia cases and samples received during the Study period. (Purposive Sampling)

STATISTICAL ANALYSIS

Mean, median, Range and standard deviation was calculated for all continuous variables. Pearson and fisher's exact chi square test was done to compare the categorical data. Mann-Whitney's test is used to calculate p value of HGB, Bilirubin, LDH and reticulocytes. Binary logistic regression analysis was used for comparing categorical data and to calculate the odds ratio with 95% confidence interval (CI). p value < 0.05 was considered significant.

INCLUSION CRITERIA

1. Patients with clinical and laboratory evidence of hemolysis with positive DAT.

2. Patients who are willing to participate in the study.

EXCLUSION CRITERIA

1. Patients with evidence of hemolysis, but DAT negative.
2. Patients who are not willing to participate in the study.

This study was carried out in the Department of Transfusion Medicine The TN Dr.M.G.R Medical University, Chennai and Department of Hematology Rajiv Gandhi Government Hospital, Chennai. All DAT requests received during the study period (i.e.) August 2017 to September 2018 were evaluated for the study eligibility.

All samples were received for DAT in Ethylene Diamine Tetra Acetic acid (EDTA) tube/Vaccutainers. Blood grouping and Rh typing was done by tube technique. Polyspecific DAT was performed by CAT using LISS Coombs ID card "DAT IgG/C3d" which can detect IgG and C3d.

The blood samples of the patients', who could potentially be included in the study, were temporarily stored at room temperature 37^0C and samples were processed within 24 hours of collection.[7]

Patients who had positive polyspecific DAT results were further evaluated by using monospecific LISS Coombs ID card "IgG, IgM, IgA, anti C3d and anti C3c " which could detect the presence of anti-IgG, anti-IgM, anti-IgA, anti-C3d and anti-C3c. If monospecific cards are positive, further IgG subtyping was performed using

anti human globulin IgG1 and IgG3 by CAT from BIORAD and this was done in two dilutions of 1:1 and 1:100.

Further, to find out the specificity of the antibody, elution studies were carried out using Glycine acid elution and Glycine acid /EDTA elution procedures. Autoadsorption was carried out in AIHA samples having IAT more than or equal that of DAT.

AIHA is further classified into Primary and Secondary AIHA based on history, laboratory and radiological results.

The degree of severity of AIHA was classified into moderate or severe based on following laboratory parameters.

1. Hemoglobin < 9gm/dl
2. Unconjugated Bilirubin >2mg/dl
3. LDH > 500 IU/ml and
4. Reticulocytes >2%

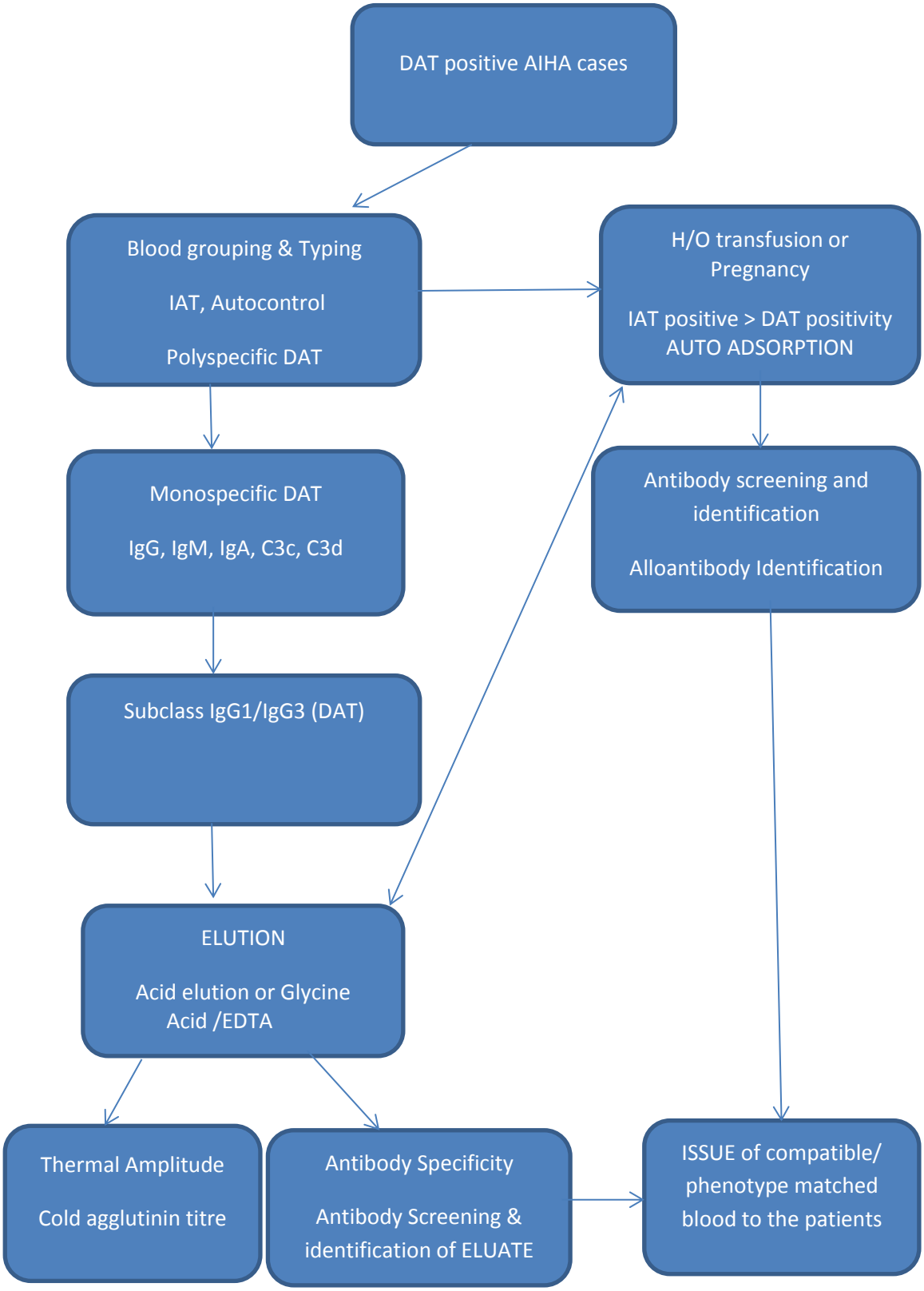
For HDFN

1. HGB < 14gm/dl and 14-18gm/dl.[160,109]
2. Uncojugated bilirubin >15mg/dl and <15mg/dl.[67,109]

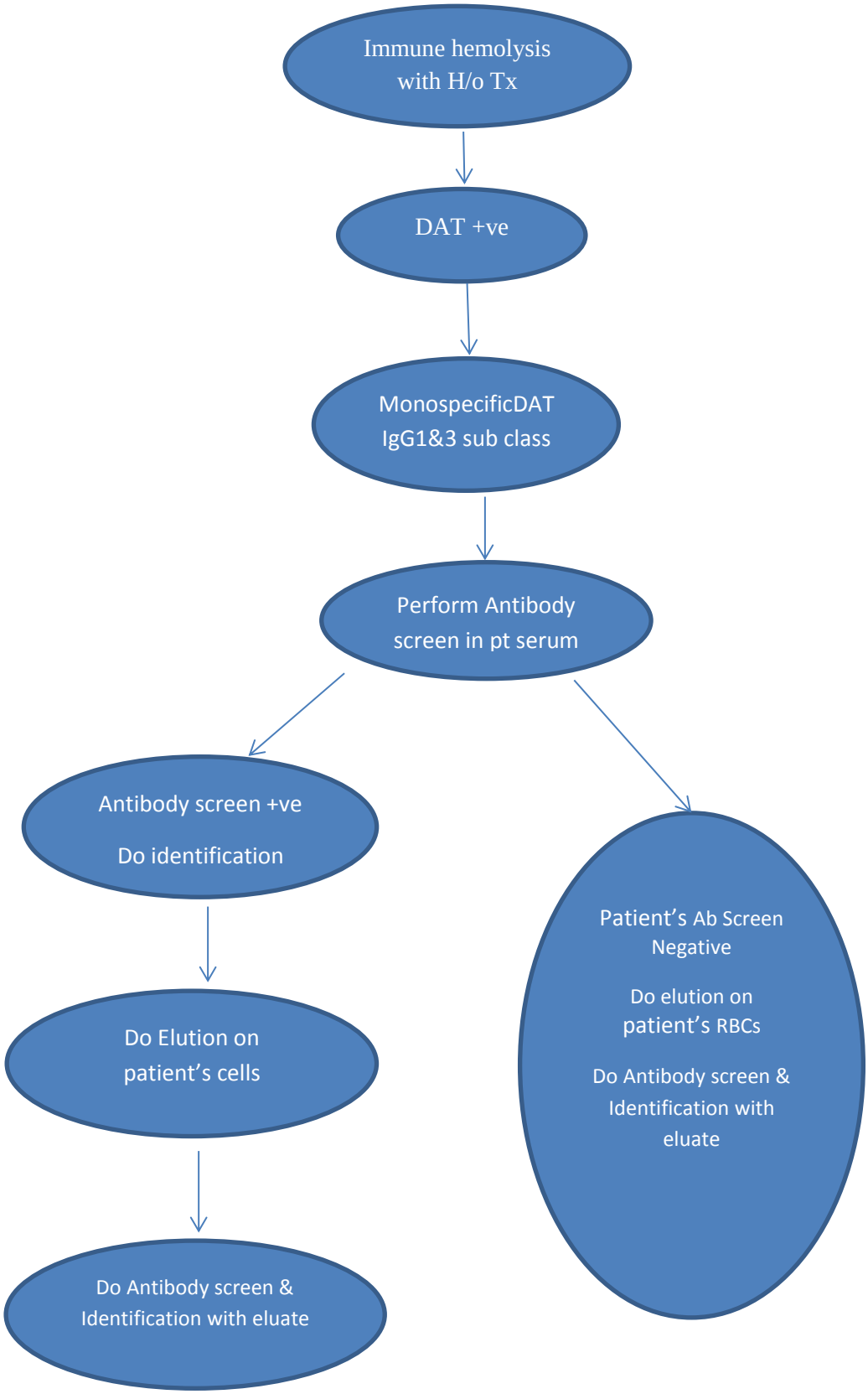
The hemolysis is classified into severe if all the above said parameters were fulfilled and classified into moderate on the basis of whether two or three of the above said laboratory parameters mentioned above are abnormal.[14,77,78]

The study algorithms are shown below:

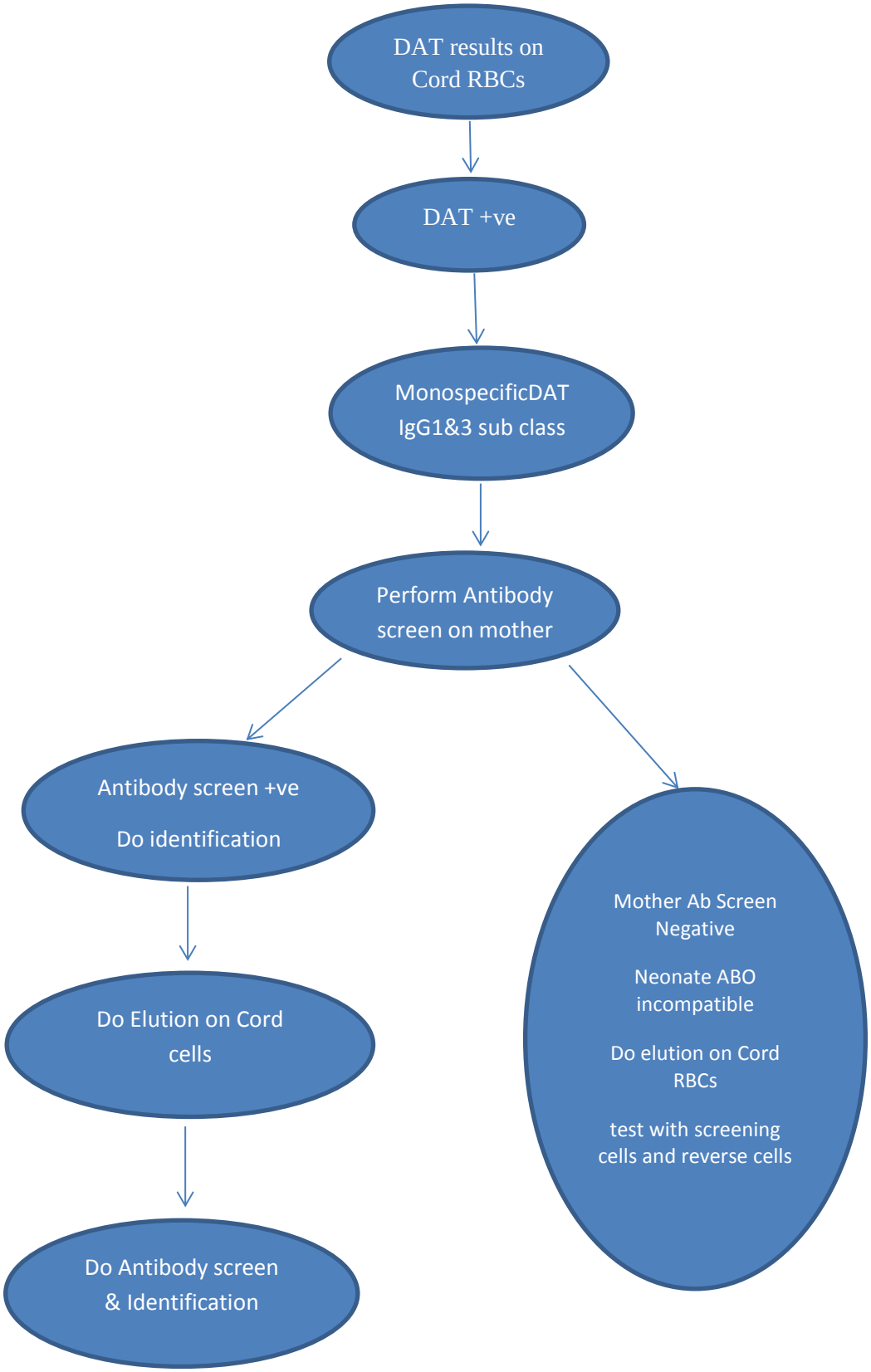
ALGORITHYM FOR DAT POSITIVE AIHA CASES



ALGORITHM FOR DAT POSITIVE DHTR CASES



ALGORITHM FOR DAT POSITIVE CORD RED BLOOD CELLS (HDFN)



LABORATORY PROCEDURES

1. Specimen collection.
2. ABO blood grouping by tube method.
3. Rh typing by tube method.
4. Direct Antiglobulin test by poly specific Anti Human Globulin (AHG) reagent by Gel Method.
5. Direct Antiglobulin Test by mono specific reagents by Gel method (anti IgG, anti IgA, anti IgM, anti C3c, anti C3d).
6. Determination of IgG subclasses by Anti-IgG1 and Anti-IgG3 reagents by Gel Method
7. Thermal Amplitude Test.
8. Cold autoantibody titration.
9. Elution studies done by Glycine Acid method.
10. Elution studies done by Glycine Acid EDTA method.
11. Determining the Specificity of Cold-Reactive Autoagglutinins.

Method 1: Sample collection procedure

Skin over the venepuncture site is cleared with 70% isopropanol and allowed to dry spontaneously. After applying tourniquet, 10ml of blood was withdrawn from antecubital vein by means of 10cc syringe and 23 gauge needle into EDTA and plain vaccutainers. Serum and red cells are separated by centrifuging at 3000rpm for 3minutes.

Method 2: Determination of ABO Group of Red Cells and Serum (Tube)

Reagents

Monoclonal anti-A, anti-B, anti-AB and A_1, B, O pooled red cells.

Procedure

1. Place 1 drop of anti-A, anti-B, anti-AB in corresponding clean, labelled test tubes.
2. To each tube, add 1 drop of a 2-5% suspension (in saline) of the red cells to be tested.
3. Gently mix the contents of the tubes; then centrifuge to 1000rpm for 1 minute.
4. Gently resuspend the cell buttons, and examine them for agglutination.
5. Read, interpret, and record the test results.
6. Compare the red cell test results with those obtained in the serum or plasma tests.

Serum Grouping

1. Add 2 or 3 drops each of serum or plasma to three
2. Clean, labelled test tubes
3. Add 1 drop of 2-5% A1 Pooled red cells to the tube labelled A1cells.
4. Add 1 drop of 2-5% B pooled red cells to the tube labelled B cells.
5. Add 1 drop of 2-5% O pooled red cells to the tube labelled O cells.
6. Gently mix the contents of the tubes; then centrifuge to 1000rpm for 1 minute.
7. Examine the serum overlying the red cell buttons for evidence of hemolysis. Gently resuspend the cell buttons, and examine them for agglutination

8. Read, interpret, and record test results. Compare serum test results with those obtained in testing red cells

Interpretation

1. Agglutination of tested red cells and either hemolysis or agglutination in tests with serum or plasma constitute positive test results. (Appendix)
2. A smooth cell suspension after re-suspension of the cell button is a negative test result.

Method 3: Determination of Rh D type (Tube)

Reagents

Monoclonal IgM, IgM & IgG (Blend), Test tubes and Centrifuge

Procedure

1. Place one drop of Monoclonal IgM in test tubes labelled D1, Positive control 1, Negative Control 1.
2. Place one drop of Monoclonal IgM & IgG (blend) in test tube labelled D2, Positive control 2, Negative control 2.
3. Add one drop 2-5% cell suspension of test cells to test tubes labelled D1 & D2.
4. Add one drop 2-5% cell suspension of known Rh D positive cells to test tubes labelled Positive Control 1& 2.
5. Add one drop 2-5% cell suspension of known Rh D Negative cells to test tubes labelled Positive Control 1& 2.
6. Mix gently and centrifuge at 1000rpm for 1 minute
7. Gently resuspend the cell button and examine for agglutination.
8. Grade reaction and record the results (test & control).

9. In case of negative reaction incubate IgM & IgG (blend) test tubes at 37˚C for 30 minutes.
10. Confirm the Negative results with weak D/Du testing.

Interpretation

1. Agglutination of tested red cells and agglutination in Positive Control constitute positive test results.

2. A smooth cell suspension after re-suspension of the cell button in tested red cells and Negative Control and after weak 'D' test is a negative test result.

Method 4: Direct Antiglobulin Testing (DAT) using LISS Coombs' Gel Card

Materials:

LISS Coombs' Gel Card, Pipette, Tips, Card Holder, ID-Centrifuge.

Principle

To detect the presence of IgG and Complement binding antibodies coated on patients' red cells. Cells coated with IgG and/or complement will show agglutination with broad spectrum AHG reagent.

Procedure

1. Label the Microtube with patient's name. Remove the Aluminium foil of as many Microtubes required by holding the ID card in upright position.
2. Pipette out 50µl of red cell suspension (0.8-1%) to appropriate Microtubes.
3. Centrifuge the ID-Card at 910rpm for 10 minutes in the ID-Centrifuge
4. Read and Record the results.(read the card from both sides).

Interpretation

Positive: Agglutinated cells forming a red line on the surface of the gel or dispersed in the column.

Negative: Compact button of cells settled on the bottom of the Microtube.

Gel test reactions are graded from 0 to 4+ (Appendix).

Method 5: Direct Antiglobulin Testing (DAT) using Monospecific Gel Card

Principle:

To detect the presence of incomplete antibodies and complement coated on patients' red cells which are IgG, IgM, IgA, C3d, and C3c using Monospecific gel card containing Anti IgG, Anti IgM, Anti IgA, Anti C3d and Anti C3c respectively.

Materials

1. ID-Monospecific card with 6 columns (Fig2 containing Anti IgG, Anti IgM, Anti IgA, Anti C3d, Anti C3c and Negative Control Microtube)
2. Micro Pipette, Micro Tips, ID-Centrifuge

Procedure

1. Label the Monospecific DAT ID-card appropriately with patients' ID/hospital ID No.
2. Remove the aluminium foil, by holding the card in upright position.
3. Add 50µl of 0.8% suspension of patients' red cells suspended in LISS to monospecific ID-Card.
4. Centrifuge the card in ID-Centrifuge at 910rpm for 10minutes.
5. Read and record the reactions. (Read the reactions on both sides of the card).
6. If the patients were noted to have IgG antibodies on monospecific DAT, Further subtyping of IgG was done.

Method 6: IgG1 and IgG3 Subclass Identification

Principle

The risk of hemolysis depends on the amount of IgG coated on the Red cells and also on the subclass of the IgG. To induce phagocytosis it is said that approximately 1000-4000 IgG1 molecules or 135-500 IgG3 molecules must coat the red cells. Therefore it is not only the presence of subclasses and also the estimation of titre levels to comprehend clinical significance.

The ID-Sub Class gel card "DAT IgG1/IgG3" has two Dilutions for both classes.

Materials

1. Subclass ID-card with 6 Microtubes (fig 3, two Microtubes having 1:1 and 1:100 dilution for both IgG1 and IgG3 respectively, one Negative Control and one Positive Control Microtubes)

2. Pipettes, Micro Tips, ID-Centrifuge

Procedure

1. Label the ID-card (Subclass) with appropriate patient ID/Hospital No.
2. Remove the aluminium foil, by holding the card in upright position.
3. Pipette 50µl of 0.8% suspension of patients' red cells suspended in LISS into each Microtube.
4. Centrifuge the card in ID-Centrifuge at 910rpm for 10minutes.
5. Read and record the reactions. (Read the reactions on both sides of the card).

Interpretation

A. Positive:

A positive reaction with the dilution of 1:1 has a sensitivity of approximately 1000 anti-IgG1 molecules and 125 anti-IgG3 molecules respectively.

A positive reaction in 1:100 dilution is indicative of higher strength of antibodies coating the RBCs.

B. Negative:

Compact button of cells settles at the bottom of Microtube.

Method 7: Thermal amplitude of autoantibodies

Materials

Test tubes, 3% to 5% patient's red cell suspension, Pipette, tips and Centrifuge.

Procedure

1. Label three test tubes as 4˚C, 22˚C, 37˚C.
2. Add 100µl of patient's serum to be tested to each of the labeled test tubes.
3. Add 50 µl of patient's 3% to 5% red cell suspension to each test tube.
4. Incubate at respective temperature for 30 minutes.
5. Centrifuge at 1000rpm for 1minute.
6. Examine each tube for agglutination and record the results.
7. Wash each tube with normal saline for 3 times.
8. Add 2 drops of AHG to each test tube and mix. Centrifuge at 1000rpm for 1minute. Examine each tube for agglutination
9. If macroscopic agglutination is not observed or if it is equivocal then examine microscopically for agglutination. Interpret the results.

Method 8: Cold Agglutinin Titer Procedure

Specimen

Serum or plasma, separated at 37 C from a sample maintained and/or allowed to clot at 37 C, or plasma, separated from an anticoagulated sample after periodic inversion at 37 C for approximately 15 minutes.

Reagents

1. A pool of two or more examples of washed group O I adult red cells
2. Normal saline (NS), pH 7.3.

Procedure

1. Prepare serial twofold dilutions of the patient's serum or plasma in NS. The dilution range should be from 1 in 2 to 1 in 4096 (12 tubes).
2. Mix 2 drops of each dilution with 1 drop of a 3% to 5% suspension of red cells.
3. Mix and incubate at 4 C for 1 to 2 hours.
4. Centrifuge the tubes for 15 to 20 seconds at 900 to 1000 × *g*, and then place the tubes in a rack in an ice water bath. Examine the tubes one by one macroscopically for agglutination, starting with the tube at the highest dilution.
5. Grade and record the results.

Interpretation

1. The titer is the reciprocal of the highest serum dilution at which macroscopic agglutination is observed.

2. Titers above 64 are considered elevated, but hemolytic anemia resulting from cold-reactive autoagglutinins rarely occurs unless the titer is ≥1000.

3. Titers below 1000 may be obtained when the autoantibody has a different specificity (eg, anti-i) or if the cold agglutinin is of the less-common low-titer, high thermal-amplitude type.

4. If the patient has a positive direct antiglobulin test (DAT) because of complement only and has clinical signs of hemolytic anemia, specificity and thermal amplitude studies should be performed.

Method 9: Elution of DAT positive Red Cells by Glycine acid Method

Principle

For identification, the antibody is separated from the red cells by elution techniques such as acid elution which is considered most suitable for elution of antibodies.

DiaCidel consists of ready to use reagents offers an easy working procedures for the elution of most common antibodies.

Materials

1. Concentrated wash solution containing Glycine-NaCl buffer
2. Elution solution containing a low pH glycine buffer with colour indicator.
3. Buffer solution containing Tris buffer with bovine albumin (1.2%)
4. Test tubes, Tube rack, Micro pipette, Micro tips
5. ID coombs' card, Antibody screening and identification Reagent Cells
6. ID-Incubator, ID-Centrifuge

Procedure

Preparation of the working solution

Dilute the concentrated wash solution to 1:10 dilution in distilled water (1part concentrate + 9 parts of distilled water).

Stability of the working solution

The working solution can be stored in a closed bottle for 6 months at 2-8˚C.

Elution

1. Wash the red cells which have given a positive DAT once with isotonic saline solution. (approximately 1ml of packed cells needed)
2. Wash 1.0ml of packed red cells 4 times with DiaCidel working wash solution.
3. Decant completely after last wash and keep part of supernatant to test for the presence of irregular antibodies
4. Add 1.0 ml of DiaCidel elution solution to 1.0 ml washed red cells.
5. Centrifuge immediately for 1 minute at 3000rpm (900g).
6. Transfer eluate into a clean test tube.
7. Add 5 drops of DiaCidel buffer solution to the eluate and mix well. Observe the formation of a blue colour, indicating that neutral pH 6.5-7.5 is reached.
8. Centrifuge the eluate for 1 minute at 3000rpm (900g) to completely remove any residual cells.
9. Eluate is ready for further testing like IAT, Antibody screening and Identification.

Method 10: Elution by glycine acid EDTA method

Principle

Acid glycine/EDTA can be used to dissociate antibody molecules from red cell membranes. The procedure is routinely used for blood typing tests or adsorption procedures. All common red cell antigens can be detected after treatment with acid glycine/EDTA except antigens of the Kell system, Bg antigens, and Er antigens. Thus, red cells treated in this manner cannot be used to determine these phenotypes.

Specimen

Red cells giving a positive direct antiglobulin test (DAT) result.

Reagents

Solution 1: A concentrated solution of sodium EDTA. 0.1% sodium azide as a preservative.

Solution 2: A low-pH glycine. This solution contains no preservative.

Solution 3: A TRIS hydroxymethyl-aminomethane solution. 0.1% sodium azide as a preservative.

Procedure

- Wash the red cells to be treated three times with isotonic saline and resuspend them into 3-5% concentration.
- Place 30 drops of washed red cells in a clean test tube.

- Centrifuge to pack the red blood cells as completely as possible and carefully remove the supernatant without disturbing the red blood cells. 3400 rpm for 1 minute should pack the red blood cells sufficiently.

- In a separate test tube, prepare the EDTA glycine-acid solution by adding 4 drops EGA solution 1 into 16 drops of EGA solution 2.

- Immediately add the freshly prepared EDTA glycine-acid solution to washed packed red blood cells and mix gently.

- Start a timer and allow the mixture to stand at room temperature for no more than 2 minutes. If the red blood cells are markedly tanned or clumped, the treatment need to be shortened in 15 second increments until a level achieved that do not result in markedly tanning or clumping of red blood cells.

- Immediately add 4 drops of EGA solution 3(1.0 M TRIS-NaCl), and mix the contents of the tube.

- Centrifuge at 3400 rpm for 30 seconds; then aspirate and discard the supernatant fluid.

- The treated red blood cells are not markedly tanned or clumped. Wash the red cells at least three times with normal saline.

- Test the washed red cells with anti-IgG. If nonreactive with anti-IgG, the cells are ready for use in blood typing or adsorption procedures. If the DAT is still positive, one additional treatment can be performed.

Method 11: Autoadsorption

Principle

Antibodies can be removed from a serum sample by adsorption. It may be possible to harvest bound antibody by elution or examination of the absorbed serum for antibody(ies) remaining after the adsorption process.

Specimen

Serum / plasma having antibody to be adsorbed.

Reagents

Red cells (Glycine acid/EDTA treated autologous) were used for Autoadsorption

Procedure

- Wash the selected red cells at least three times with saline.
- After the last wash, centrifuge the red cells at 800 to 1000 × g for at least 5 minutes, and remove as much of the supernatant saline as possible. Additional saline may be removed by touching the red cell mass with a narrow piece of filter paper.
- Combine appropriate volumes of the packed red cells and serum, and incubate at the desired temperature for 30 to 60 minutes.
- Mix the serum or cell mixture periodically throughout the incubation phase.

- Centrifuge the red cells at 800 to 1000 × *g* for 5 minutes to pack cells tightly. Centrifuge at the incubation temperature, if possible, to avoid dissociation of antibody from the red cell membranes.

- Transfer the supernatant fluid, which is the adsorbed serum, to a clean test tube. If an eluate is to be prepared, save the red cells.

- Test an aliquot of the adsorbed serum, preferably against a reserved unused aliquot of the red cells used for adsorption, to see if all antibody has been removed.

Interpretation

If reactivity remains, the antibody has not been completely removed. No reactivity signifies that antibody has been completely adsorbed.

Notes

1. Adsorption is more effective if the area of contact between the red cells and serum is large. Use of a large-bore test tube (13 mm or larger) is recommended.

2. Multiple adsorptions may be necessary to remove an antibody completely; however, each successive adsorption increases the likelihood that the serum will be diluted and unadsorbed antibodies weakened.

3. Repeat adsorptions should use a fresh aliquot of red cells and not the red cells from the earlier adsorption.

4. Enzyme pretreatment of adsorbing red cells can be performed to increase antibody uptake for enzyme-resistant antigens.

Method 12: Determining the Specificity of Cold-Reactive Autoagglutinins

Principle

Cold-reactive autoagglutinins are usually IgM, which binds to red cells in the lower temperature of the peripheral circulation and causes complement components to attach to the red cells. As the red cells circulate to warmer areas, the IgM dissociates but the complement remains.

Specimens:

1. Serum or plasma, separated at 37 C from a blood sample maintained and/or allowed to clot at 37 C, or plasma, separated from an anticoagulated sample after periodic inversion at 37 C for approximately 15 minutes.

2. Test red cells of the following phenotypes
    1. A pool of two or more examples of adult group O I adult red cells;
    2. Group O i cord red cells.
    3. The patient's own (autologous) red cells, washed at least three times with 37 C saline.
    4. Red cells of the same ABO group as the patient, if the patient is not group O. If the patient is group A or AB, use both A1 and A2 cells.
    5. Saline

Procedure

- Prepare serial twofold dilutions of the serum or plasma in saline. The dilution range should be from 1 in 2 to 1 in 4096 (12 tubes), and the volumes prepared should be more than the total volume needed to test all of the desired red cells. For example, diluting 0.4 mL of serum with 0.4 mL of saline would be sufficient to test three red cell examples.

- Label a set of 12 tubes with the dilution (eg, 2, 4, 8, etc) for each kind of red cells to be tested (eg, adult, cord, autologous).

- Dispense 2 drops of each dilution into the appropriate tubes.

- Add 1 drop of a 3% to 5% saline suspension of each red cell sample to the appropriate set of tubes.

- Mix and incubate at room temperature for 30 to 60 minutes.

- Centrifuge for 15 to 20 seconds at 900 to 1000 × g. Examine the tubes for agglutination, starting with the set of tubes at the highest dilution for each cell tested (ie, read all the tubes for each dilution as a set). Grade and record the results.

- Incubate the tubes at 4˚ C for 1 to 2 hours.

- Centrifuge for 15 to 20 seconds at 900 to 1000 × g. Immediately place the tubes in a rack in an ice water bath. Examine the tubes as in step 6. Grade and record the results.

Interpretation

Red Cells	**Antibody Specificity**				
	Anti-I	**Anti-i**	**Anti-I^T**	**Anti-IH**	**Anti-Pr**
O I Adult	+	-	0/↓	+	+
O i Cord	-	+	0/↓	↓	+
Autologous	+	0/↓	0/↓	↓	+

RESULTS

Total of 57 cases of DAT positive samples were included in the study during the study period from August 2017 to September 2018.

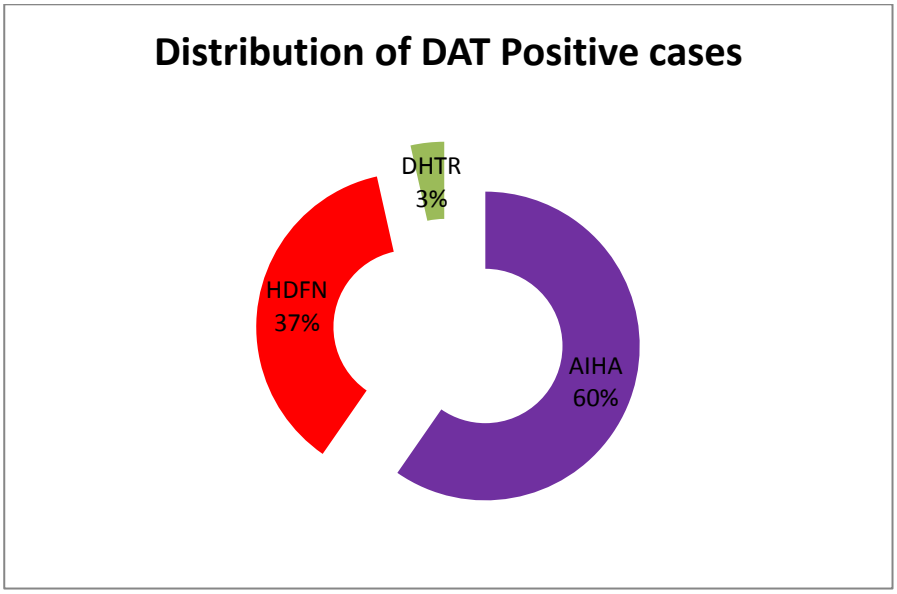
On analysing the cause of DAT positivity the patients belonged to different categories. The patients were belonged to

- Autoimmune Hemolytic Anemia,
- Hemolytic Diseases of Fetus and Newborn and
- Delayed Hemolytic Transfusion Reaction

All these patients were included in the study, since the distributions of these cases were presented with features of hemolysis and Direct Anti Globulin Test positivity.

Distribution of DAT positive cases

AIHA 34, HDFN 21, DHTR 2



Autoimmune hemolytic anemia

Out of 34 AIHA patients 25 patients were found to have WAIHA, 6 had CAS and 3 had mixed type of AIHA.

Type of AIHA

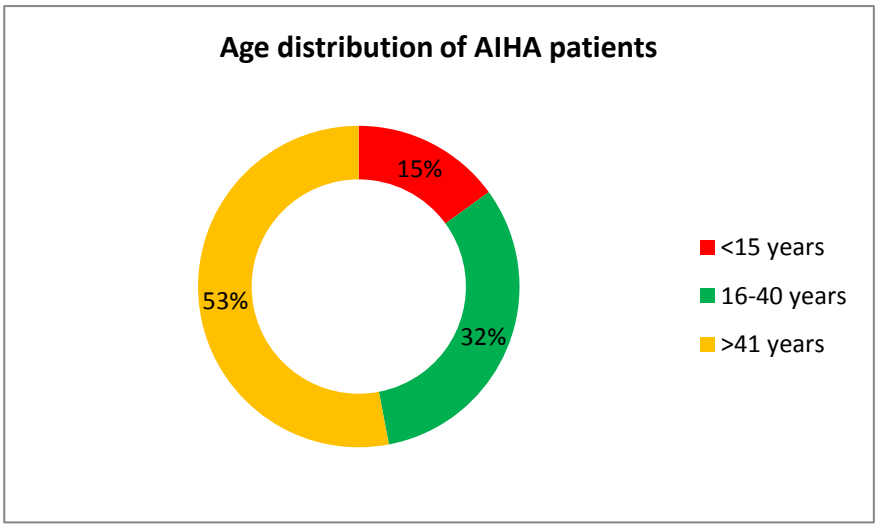
Out of 34 patients, 20 (59%) patients were diagnosed to have primary AIHA and the remaining 14 (41%) patients had secondary AIHA.

Causes of secondary AIHA are,

1. Autoimmune Disorders (SLE=4; RA=1)
2. Lymphoproliferative disorders (n=3)
3. Infections (n=1)
4. Sickle cell anemia(n=2)
5. Ovarian tumours(n=2) and
6. Cancer cervix (n=1)

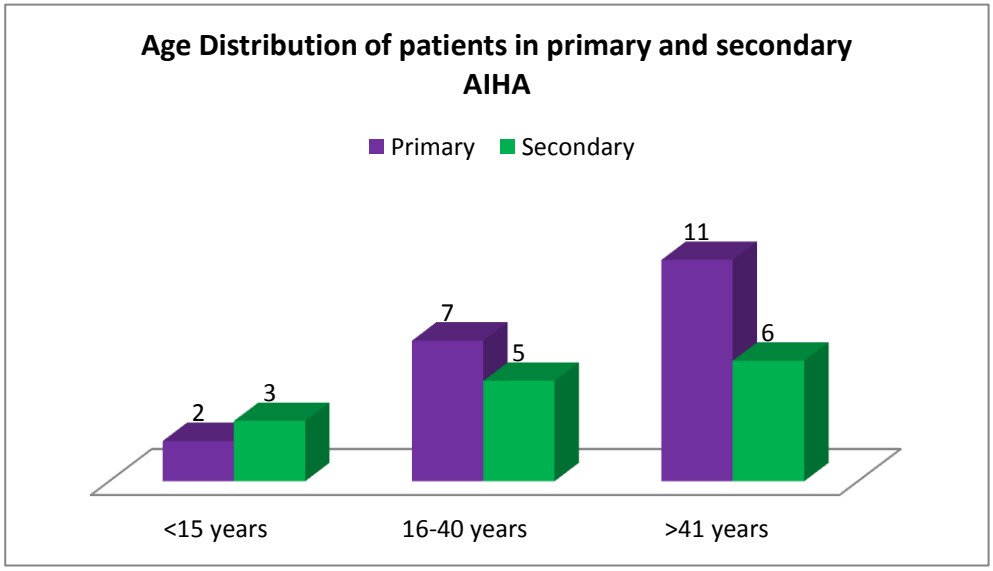
Age Distribution of AIHA Patients

The age distribution of AIHA patients ranged from 3 years to 79 years with a median age of 38 years.



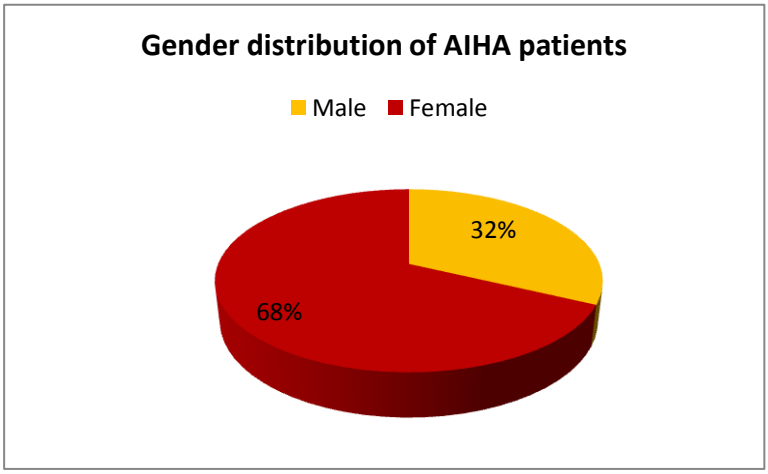
Age Distribution of Patients in primary and secondary AIHA

When comparing the age distribution of between primary and secondary AIHA, median age was 40 years in primary (Range 3-79years) and 26 years in secondary AIHA (Range 5-70 years). Primary AIHA was most predominantly seen in >41 years of age (58%) and Secondary AIHA was more common among < 30 years of age (57%).



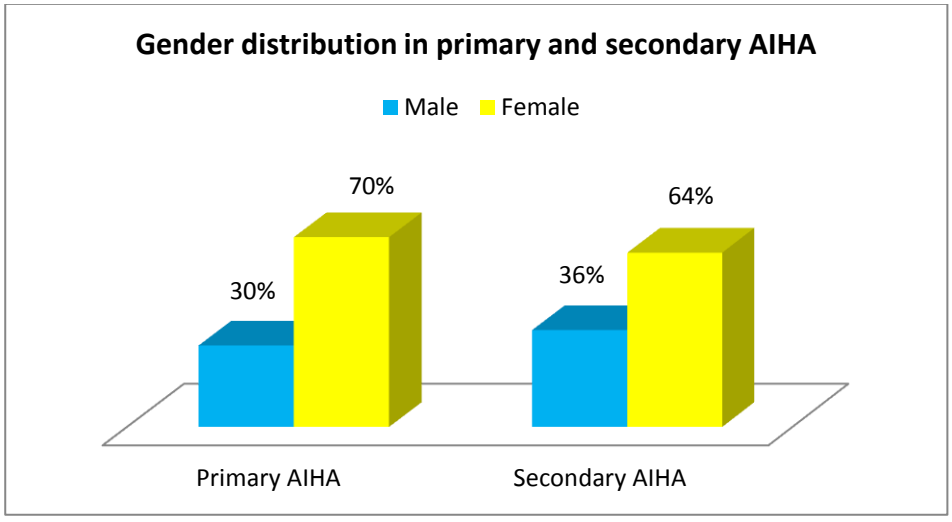
Gender distribution

In our study of 34 AIHA patients, 11 (33%) were males and 23(67%) were females with overall ratio of 1:2 (11:23)



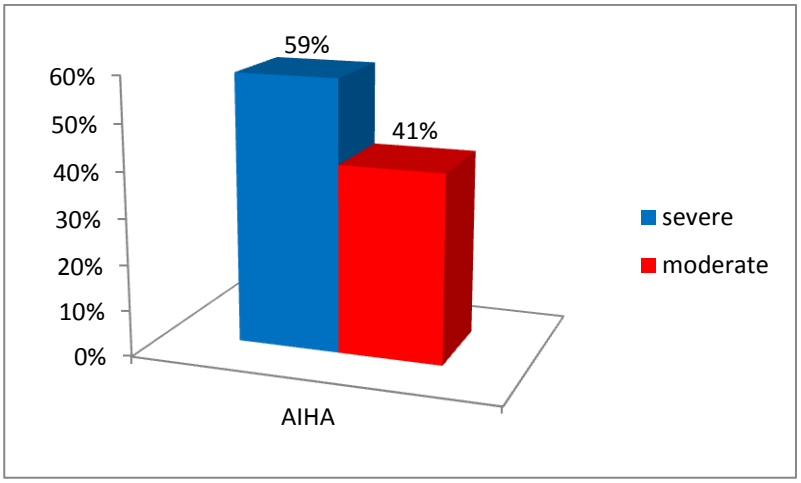
Gender distribution in primary and secondary AIHA

While comparing the gender distribution among primary and secondary AIHA patients, Male to Female ratio was noted to be 1:2.2 in primary AIHA and 1:1.8 for secondary AIHA.



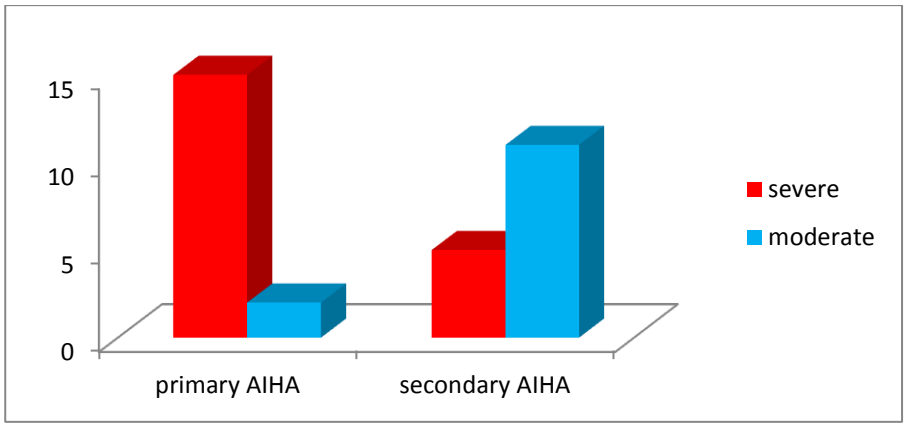
Severity of AIHA

Hemolysis was classified into Severe, Moderate based on study criteria laid down by Wheeler et al and Das ss et al. out of 34 AIHA patients, 20 (59%) patients were belonged to severe hemolysis, 14(41%) patients had moderate hemolysis as shown in the picture below.[77,14]



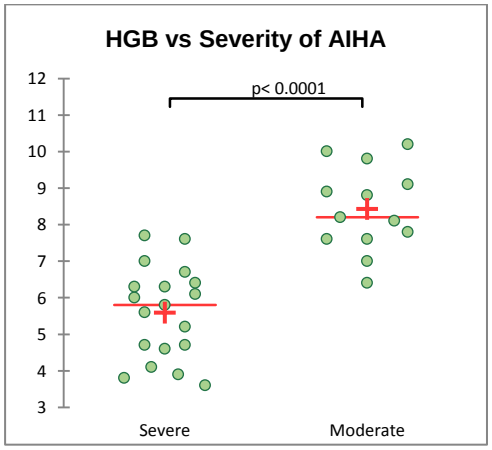
Severity of hemolysis in primary and secondary AIHA patients

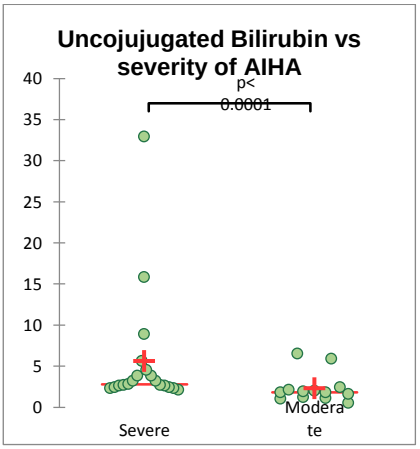
Among 20 AIHA patients with severe hemolysis, 12 had primary AIHA and 8 had secondary AIHA. The association of primary AIHA with severe Hemolysis found to be statistically significant with p <0.006 (OR=13.5, 95% CI 2.15-84.70). Moderate hemolysis was common among secondary AIHA patients.



HGB and Bilirubin values in primary and secondary AIHA patients

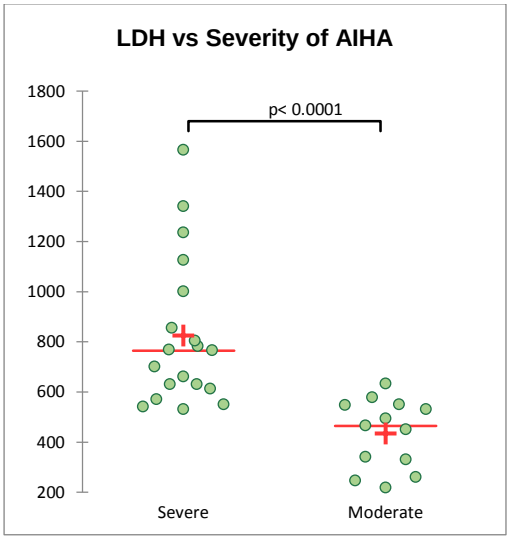
Hemoglobin and Bilirubin were assessed in AIHA patients with severe and moderate hemolysis by unpaired T test. There is statistically significant difference in mean hemoglobin in patients with severe and moderate hemolysis. The mean hemoglobin of severe hemolysis is 5.6gm/dl and of moderate hemolysis is 8.2gm/dl. (p <0.0001).

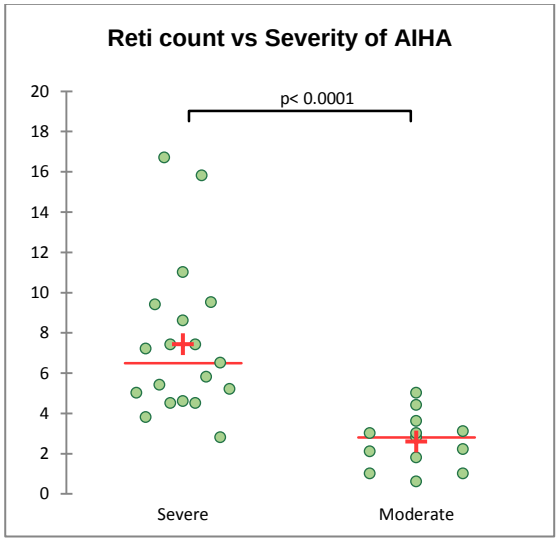




Reticulocytes and LDH values in severe and moderate hemolysis

Values of Reticulocytes and LDH Values were assessed in AIHA patients with severe and moderate hemolysis by Mann Whitney test and they are statistically significant with a p value of <0.001.

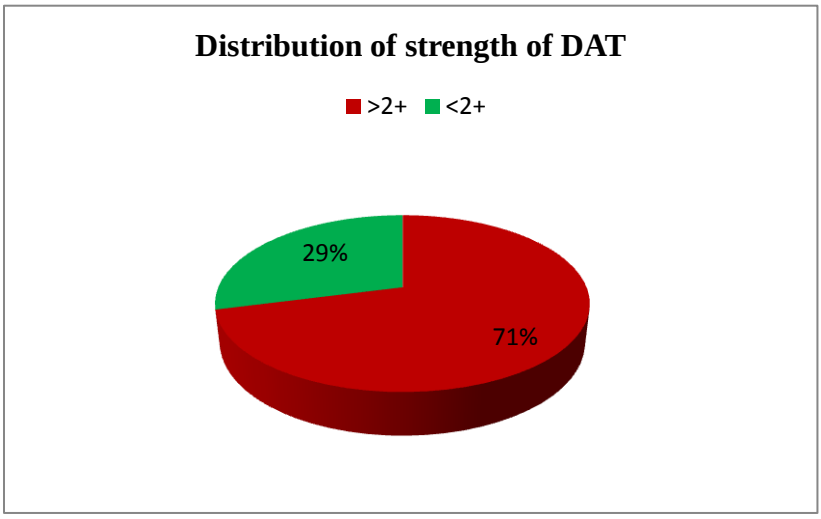




Immunohematological Parameters

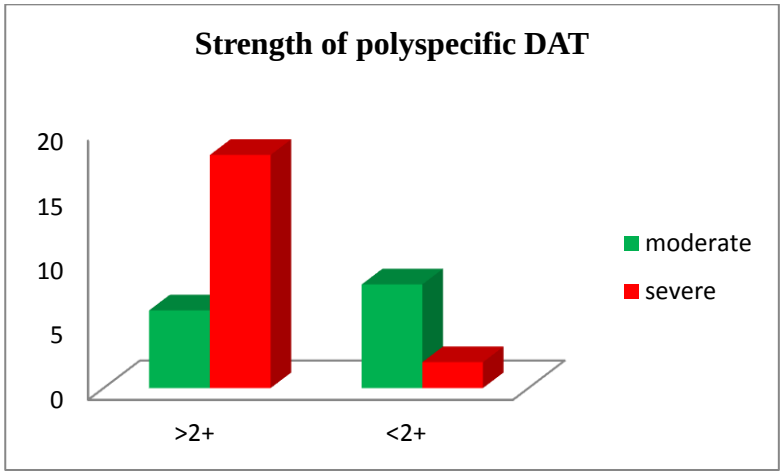
Distribution of polyspecific DAT positivity among AIHA patients

Out of 34 AIHA patients, 24 (71%) patients were having DAT strength of >2+ and remaining 10 (29%) patients had DAT strength of <2+.



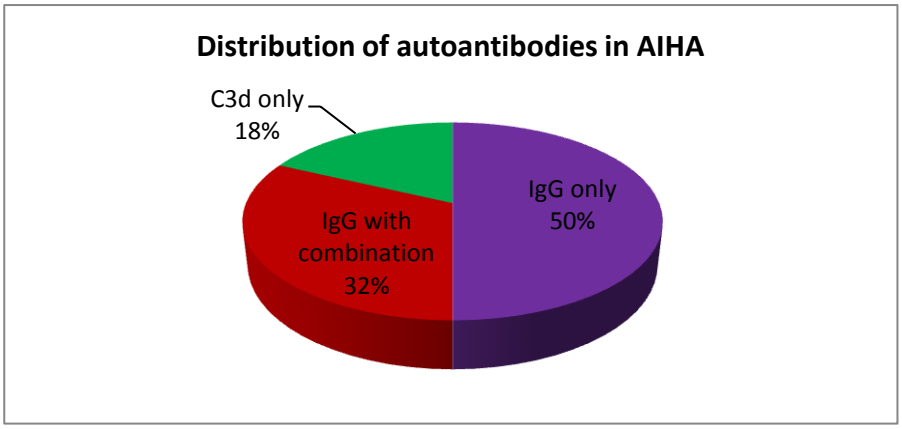
The strength of polyspecific DAT with severity of hemolysis

We found that Among 24 AIHA patients with >2+ DAT strength 18 (75%) of had severe hemolysis, but only 2 (10%) out of 10 AIHA patients with <2+ DAT strength had severe hemolysis. The chi-square test showed that the patients with DAT strength more than 2+ were more likely to have severe hemolysis compared to patients having 2+ or less DAT reactions. **This correlation was statistically significant with a p value of < 0.003.**



Distribution of autoantibodies in AIHA

Among the 34 patients of AIHA, multiple antibodies (ie; IgG with other antibodies or complement) were found in 17 (50%) patients, IgG alone was found in 11 (32%) patients and C3d alone was identified in 6 (18%) of AIHA patients.



The various combinations of Autoantibodies (Monospecific DAT) in the study population

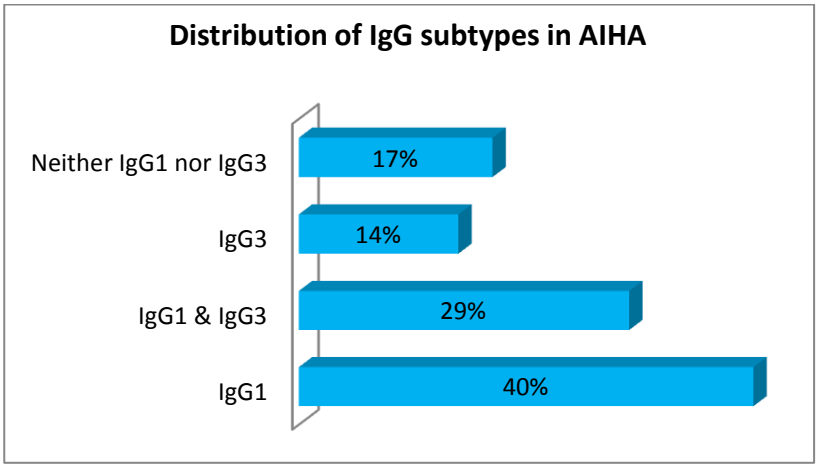
Autoantibody Combinations	Severe	Moderate
	N=20	N=14
IgG only	3	8
IgG & C3d	11	1
IgG & IgA	2	0
IgG, IgM & C3d	1	0
IgG, IgM, IgA &C3d	2	0
C3d only	1	5

Comparison of IgG only and IgG with multiple Antibodies with or without complement

Among 20 AIHA patients with severe hemolysis, 16 (80%) had combination of antibodies in comparison to 3 (15%) patients who had solitary IgG antibody. 1 (5%) severe hemolysis patient was having only C3d. The severity of hemolysis is statistically significant in AIHA patients having autoantibodies (IgG with combination) with a **p value of 0.002.**

Distribution of IgG subtypes

Out of 28 patients with IgG, 11 of them had solitary IgG antibody and 17 of them had IgG with combination of other autoantibodies. IgG1 was the most common subtype present in 11 (40%) patients, followed by IgG1 and IgG3 which was present in 8 (29%) patients and solitary IgG3 was found in 4 (14%) patients with AIHA. In our study 5 (17%) patients had neither IgG1 nor IgG3.



Correlation of IgG subtypes with severity of hemolysis

The presence of both IgG1 and IgG3 has a significant effect on severity of hemolysis. A total of 8 patients were noted to have **both IgG1 and IgG3** in which 7 (87%) had severe hemolysis in par with 1 (13%) moderate hemolysis. A similar impact was noted in patients with **solitary IgG1** [95% CI= 25.0570 to 88.9932; P = 0.0042]. Among 11 AIHA patients with only IgG1 autoantibody 10 (90%) had severe hemolysis and 1 (10%) 95% [CI= 39.2760 to 91.4404; P = 0.0002] were having moderate hemolysis. **Both of these associations were statistically significant.**

Among 4 AIHA patients who had IgG3 subclass, 3(75%) had moderate hemolysis and 1 (25%) had severe hemolysis. In 5 AIHA patients who had neither IgG1 nor IgG3, 4 (80%) had moderate hemolysis and 1 (10%) had severe hemolysis.

Correlation of IgG subtypes with combination of other autoantibodies

IgG subtypes in combination with other antibodies and/or complement was found in 17 AIHA patients.

IgG subtypes with other Antibodies	moderate	severe	total	p value
IgG1	0	10	10	0.126
IgG1 and IgG3	1	4	5	

I group of 10 AIHA patients were noted to have IgG1 in combination with other autoantibodies and/or complement. All of them had severe hemolysis.

II group of 5 AIHA patients who had IgG1 and IgG3 in combination with other autoantibodies and/or complement, 4 had severe hemolysis in comparison to 1 patient with moderate hemolysis. Logistic analysis showed the patients with severe hemolysis were associated with the presence of both IgG1 alone and IgG1 with IgG3, however this is not statistically significant.

The association of IgG subclass in combination with other autoantibodies and/or complement to severe hemolysis was **not statistically significant** among the study population.

Correlation of complement fixation in AIHA patients with IgG1 and/or IgG3 subclass

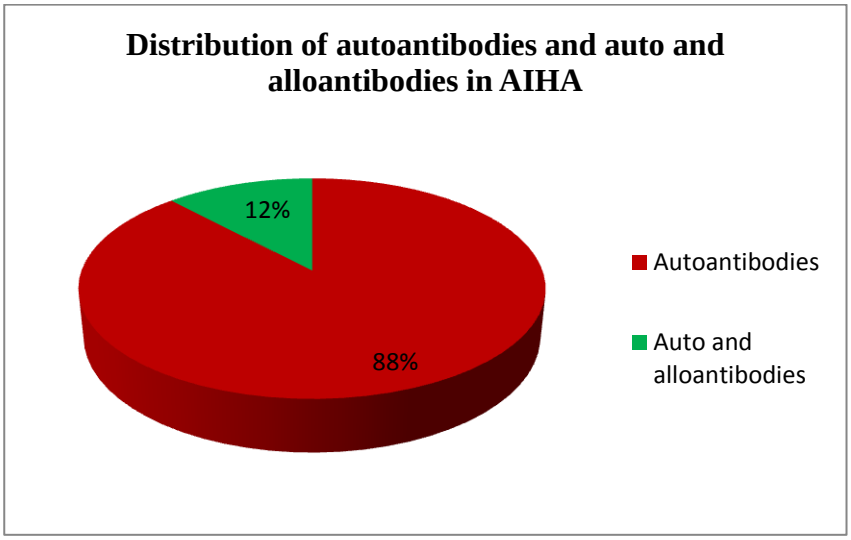
Among AIHA patients the impact of complement fixation with IgG1 and/or IgG3 subclass were studied. In the study population, 11 patients were having IgG1 and/or IgG3 without complement fixation as compared to 13 patients who had complement fixation.

	moderate	**Severe**	**Total**	**p value**
IgG1 &/ IgG3	8	3	11	0.000
IgG1 &/ IgG3 with complement	1	14	15	

This association between IgG1 and/or IgG3 and complement fixation is statistically significant with a p value of 0.000.

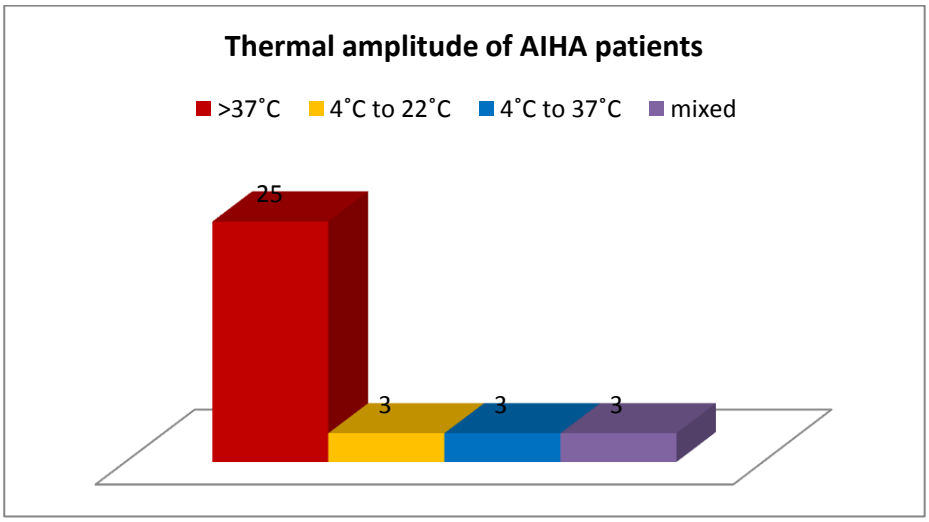
Distribution of Auto and Alloantibody among AIHA patients

Out of 34 AIHA patients 30 (88%) had autoantibodies and 4 (12%) patients had both auto and alloantibodies.



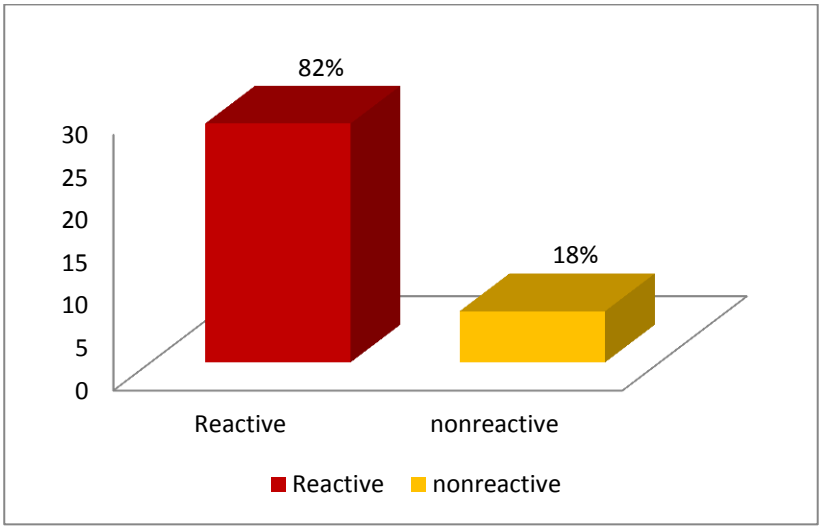
Thermal Amplitude of auto antibodies

Out of 34 AIHA patients, 25 warm autoantibodies reacted at 37˚C, among 6 Cold AIHA 3 had thermal amplitude of 4˚C to 22˚C and 3 had wide thermal amplitude of 4˚C to 37˚C. In 3 Mixed AIHA cold autoantibodies had thermal amplitude of 4˚C to 22˚C and warm autoantibodies reacted at 37˚C.

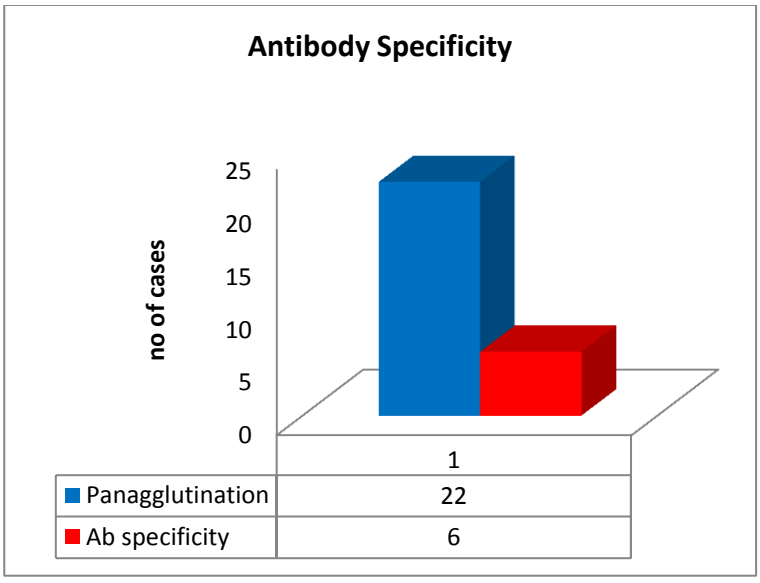


Acid Elution in AIHA patients

Among the 34 DAT positive samples, Elution was done in all AIHA patients, who had IgG autoantibody alone, IgG with other autoantibodies and/or complement and complement alone. Out of 34 elutions, 28(82%) samples were reactive with RBC reagent panel and 6 (18%) samples were nonreactive with the same.



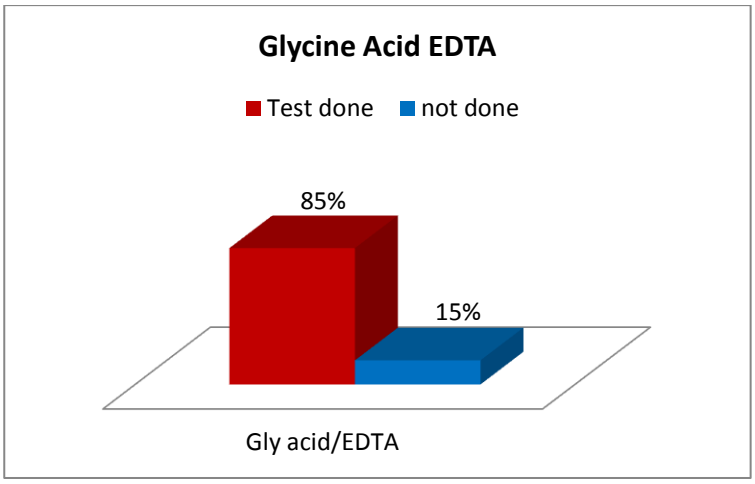
All the reactive eluates belonged to either warm or mixed AIHA patients and the nonreactive eluates belonged to patients with CAS.



Among 28 reactive eluates 22 were showing panagglutination, in which 21 (73%) were WAIHA with, 1 (4%) was due to mixed AIHA and 6 (23%) AIHA samples showed the presence of autoantibody specificity as anti-e which was confirmed with serum studies (4 were due to WAIHA and 2 were mixed AIHA).

Elution with Glycine Acid EDTA

Among 34 patients with AIHA Glycine Acid EDTA was carried out in patients who are diagnosed case of AIHA and in patients with h/o previous transfusion more than 3 months. This implies that Glycine acid EDTA was done in 29 patients of AIHA and test is not done in 3 cases of CAS (thermal amplitude of 4˚C to 22˚C) and 2 patients with a recent history of transfusion(< 7days).



Glycine Acid EDTA rendered 18 samples of DAT completely negative and in the remaining 11 AIHA cases the strength of DAT is reduced than its original strength.

Autoadsorption

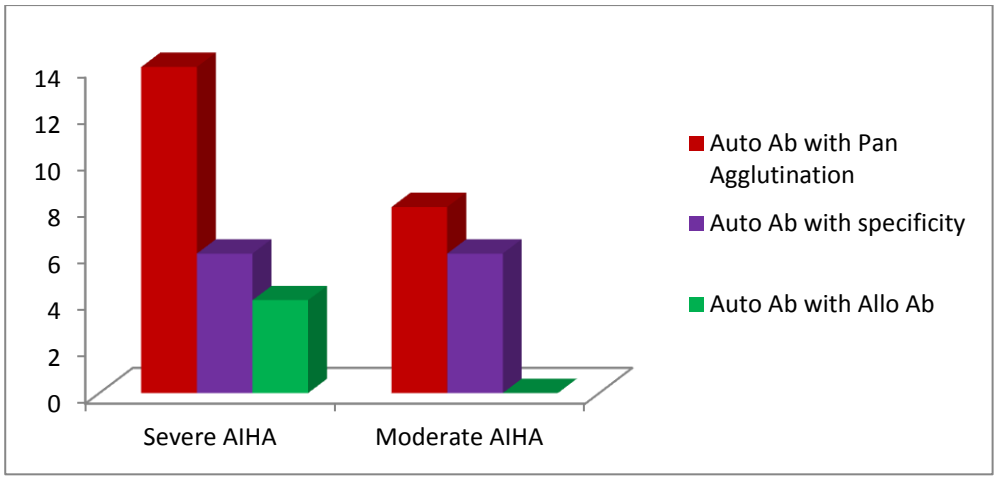
Among 28 AIHA patients, 7 patients whom had previous history of blood transfusions. The strength of IAT and DAT were correlated to rule out the presence of alloantibody. The Autoadsorption was done in 5 AIHA patients with a history of

blood transfusions more than 3 months and having IAT strength more than or equal to the strength of DAT. The Autoadsorption revealed the presence of clinically significant alloantibodies in 4 out of 5 patients and one patient was having AIHA only. (Autoadsorption done after treating the DAT positive autologous red cells with glycine acid EDTA and rendering them DAT negative).

Identified autoantibodies and alloantibodies by elution and/or absorption and elution

Out 24 AIHA patients with only autoantibodies 18 (55%) had panagglutination and 6 AIHA patients were found to have specificity against Rh antigen (anti-e).

6 Cold AIHA patients with specificity against I antigen (anti-I) 1 had severe haemolysis; in 5 patients there was moderate haemolysis.



Alloantibodies were identified in 4/20 (20%) in relation to AIHA patients with severe hemolysis and all of them had previous history of transfusion. 3 of 4 patients had single clinically significant alloantibody and 1 of 4 had multiple clinically significant alloantibodies. **The association between transfusion and alloantibody formation is statistically significant with a p value of 0.000.**

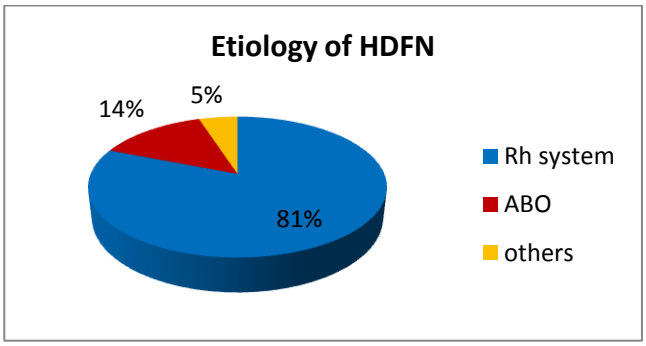
Auto and Alloantibodies vs hemolysis

	Severe hemolysis	**Moderate hemolysis**
Autoantibodies with Panagglutination	11/20	8/13
Autoantibodies with Specificity	Anti-e (4/6) Anti-I (1/6)	Anti-e (2/6) Anti-I (5/6)
Autoantibodies with Alloantibodies	Anti-C, Anti-c, Anti-E, Anti-Jka,s,Fya	0

II. Hemolytic Diseases of Fetus and New-born (HDFN)

II. 1: Etiology

Out of 21 cases of DAT positive HDFN cases received 17 were due to Rh, 3 were due to ABO HDFN and 1 is due to others (Anti-M).



II. 2: Correlation of Hyperbilirubinemia (TSB >15mg/dl) with HDFN

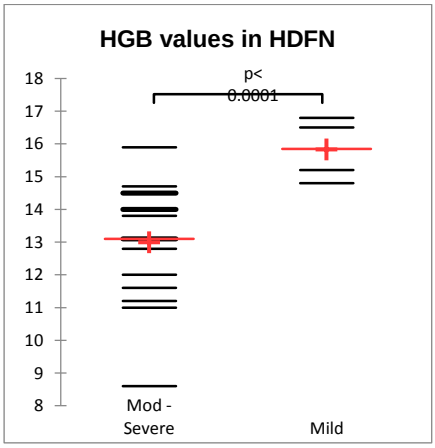
From a total of 21neonates, 16 (76%) have with hyperbilirubinemia TSB >15mg/dl, in which 5 infants needed ET and 11 were treated with phototherapy. The remaining 5 Infants who had mild hyperbilirubinemia TSB < 15mg/dl needed no intervention.

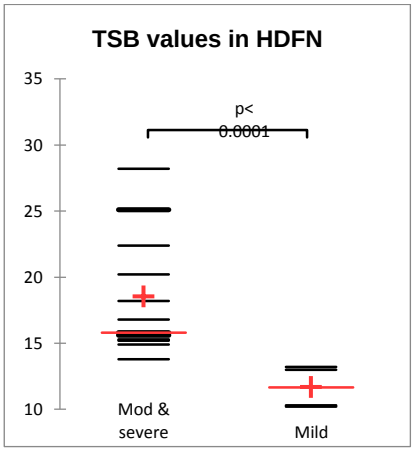
There is statistically significant correlation between hyperbilirubinemia with the management of HDFN and has a p value of < 0.000. (Mann-Whitney test / Two-tailed test)

Correlation of HGB with severity of HDFN

Hemoglobin was assessed in patients with moderate to severe HDFN and mild HDFN by unpaired T test. The mean hemoglobin of moderate to severe hemolysis is 13.46gm/dl and of mild HDFN is 16.04gm/dl.

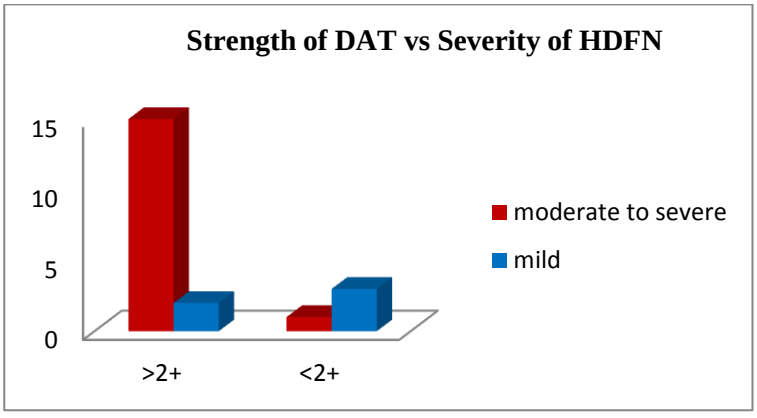
There is statistically significant Correlation in hemoglobin between patients with moderate to severe hemolysis and mild hemolysis (p <0.0013).





II. 3: Correlation between Strength of DAT with severity of HDFN

Out of 16 neonates with Moderate to severe HDFN 15 neonates had >2+ DAT strength and 1 neonates had <2+as strength of DAT and among 5 neonates having<2+ as strength of DAT, 4 of them presented with mild HDFN had 1 neonates were having Moderate to severe HDFN.



There is a statistically significant Correlation between Strength of DAT with severity of HDFN with a p value of 0.012.

II.5: Specificity of DAT in HDFN

Out of 21 cases of HDFN (100%) all of them had anti-IgG only in Monospecific DAT.

II. 6: Distribution of IgG subtypes

Out of 21 HDFN cases, IgG1 was identified in 12 neonates; IgG1 & IgG3, IgG3 and neither IgG1 nor IgG3 were seen in 3 neonates in each group.

Distribution of IgG subtypes

IgG Subtypes	Moderate to severe HDFN	Mild HDFN	Total
	N=16	N=5	N=21
IgG1	10	2	11
IgG1 & IgG3	3	0	6
IgG3	1	2	3
Neither IgG1 nor IgG3	2	1	1

II. 7: Correlation of IgG subclass with severity

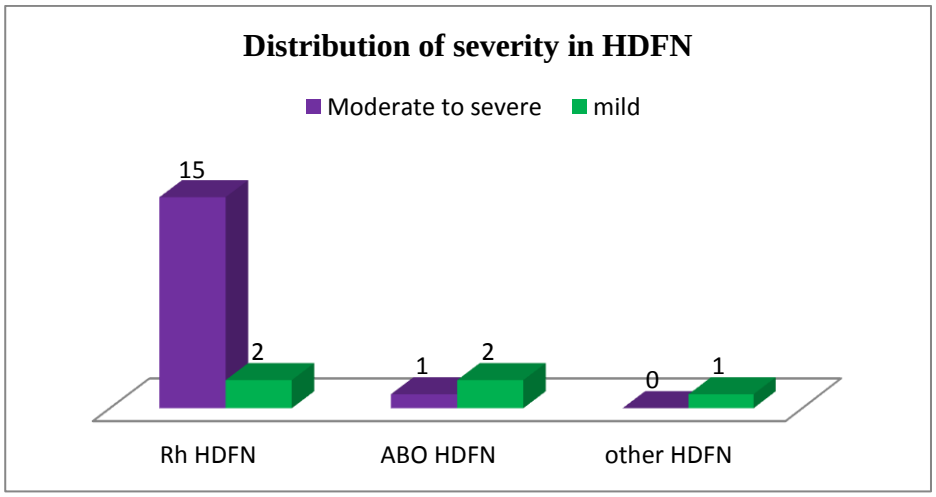
Among 21 neonates with HDFN, 11 had IgG1 (9 moderate to severe and 2 mild HDFN); 6 neonates had IgG1 & IgG3 subtypes all of them had Moderate to severe HDFN, 3 neonates were having IgG3 subtype in which 2 had severe HDFN and 1 had mild HDFN; finally in the group of neither IgG1 nor IgG3 1 neonate had mild HDFN.

IgG Subtypes	Mild	Moderate to severe
IgG1, IgG1 & IgG3	2	15
IgG3, Neither IgG1 nor IgG3	3	1

There is a statistically significant correlation between the presence of IgG1, IgG1 and IgG3 antibodies and moderate to severe HDFN with a p value of < 0.012

II. 9: Correlation of hemolysis with Rh HDFN and ABO HDFN

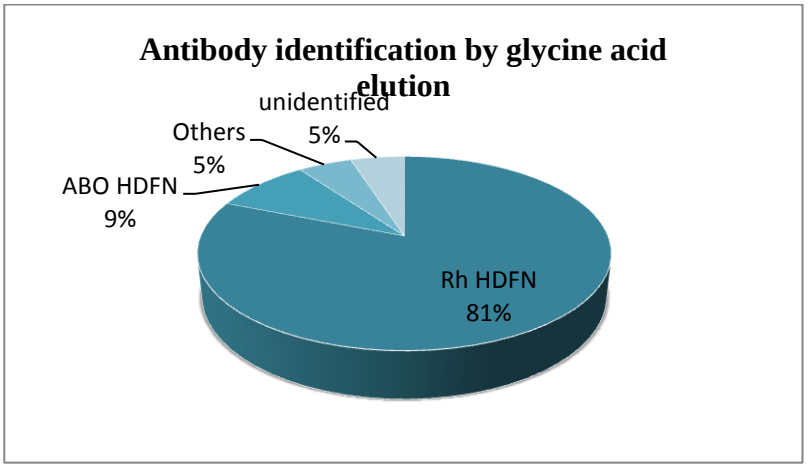
Out of 17 Rh HDFN neonates, 15 had moderate to severe HDFN and 2 had mild HDFN. Out 3 ABO HDFN neonates in this present study 1(33%) neonate had moderate to severe HDFN and 2 (67%) neonates had mild HDFN.



However, there is significant correlation between Rh HDFN and severity of hemolysis with a p value of 0.012.

II. 10: Acid Elution in HDFN

Among the 21 neonates acid elution was done in all the infants. 20 eluates were reactive and 1 eluate of ABO HDFN was nonreactive.



In this study there is a statistically significant correlation between the DAT positive neonates and antibody identification in the eluate with a p value of <0.0001.

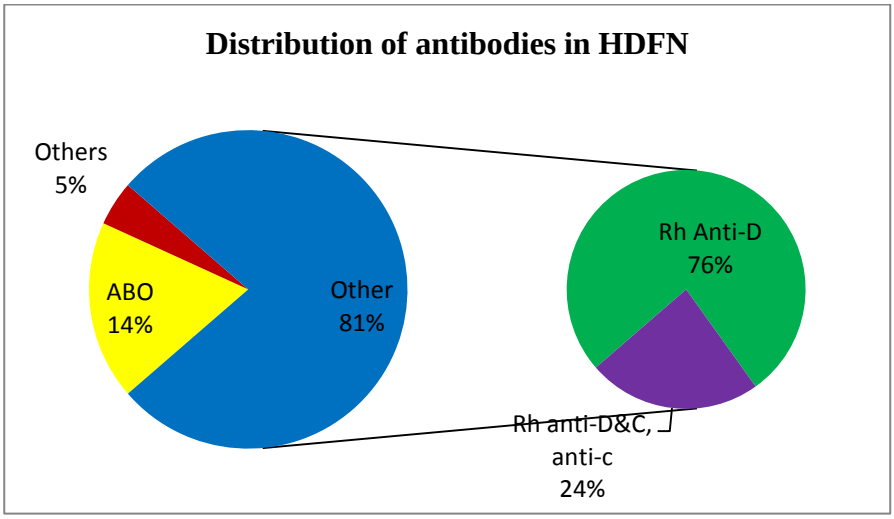
Glycine Acid EDTA Elution in HDFN

Out of 21 HDFN neonates all under gone Glycine Acid EDTA DAT was negative in and Rh phenotyping along with other specific phenotypes were identified. However, Kell antigens (K,k) were destroyed by this elution method.

Probable Phenotype	No. of neonates
D, C, c, e,	10
D, C, c, E, e	01
D, c, e	04
D, c, E, e,	02
D, C, c, e, M, N, s	01
D, c, E, e, A	01
D, C, c, e, A	01
D, C, c, e, B	01

Antibodies found in neonates with HDFN

Out of 17 Rh HDFN neonates Anti-D was found in 13 (76%) neonates, anti-D & anti-C was found in 2 (12%) neonates, anti-c was found in 2 (12%) neonates only. Among 3 ABO HDFN Anti-A was found in 1 Neonate and Anti-B was found in 1 neonate and among other blood group antigens anti-M was found in 1 neonate.



III. Delayed hemolytic / serological transfusion reaction

III. A. The strength of polyspecific DAT in DHTR

Out of 2 cases 1 had 2+ in polyspecific DAT and 1 had 1+ reaction in polyspecific DAT.

III. B. Features Of Hemolysis

	HGB gm/dl	TSB mg/dl	LDH IU/ml	Retic
Patient 1	6.3	2.8	615	4%
Patient 2	7.4	2.4	532	2%

III. C. Distribution of antibodies by monospecific DAT

Out of 2 cases of delayed hemolytic transfusion reactions both of them had IgG and C3d in their coated RBCs identified by monospecific DAT.

III. D. Distribution of IgG subtypes

Both patients of delayed hemolytic transfusion reactions had IgG1 subclass which was identified by IgG subclass card.

IV. E. Elution in DHTR

In Acid elution of both DAT positive DHTR samples we found the presence of Anti-Jk^a and anti-c both are clinically significant antibodies. However Anti-c was identified in 1 patient's serum also.

Glycine Acid EDTA Elution

Glycine Acid EDTA Elution was not performed in both the DHTR patients since they had h/o transfusion 6 days and 10 days back respectively.

Elution of DAT positive samples in overall view

Table 1: Comparison of antibodies in serum and Eluate

Antibody(-ies)	Number	% of Total
A. In serum		
Alloantibody	19 [17 Rh HDFN, 1 other(Anti-M HDFN) & 1 DHTR]	33.33%
Warm autoantibody	08 (6 anti-e & 2 panagglutinins)	14.03%
Warm autoantibody plus Alloantibody	04 (DAT & AC +ve and h/o tx present > 3months)	7.02%
Cold and Mixed Autoantibody	06 (3 CAS + 3 mixed AIHA)	10.54%
No antibodies (antibody detection test negative in serum)	20 (16 AIHA, 3 HDFN & 1 DHTR)	35.08%
Total	57	100%
B. Elute		
Alloantibody	20 [17 Rh HDFN, 1 other(Anti-M HDFN) & 2 DHTR]	35.1%
Warm autoantibody	21 (6 anti-e & 15 Panagglutinins)	36.8%
Nonreactive	07 (6 CAS & 1 ABO HDFN)	12.2%
Cold and Mixed Autoantibody	03 (All 3 Mixed AIHA)	5.3%
Anti-A or anti-B	02 (1anti-A & 1 anti-B)	3.5%
Warm autoantibody plus Alloantibody	04 [1 anti-c,1 anti-C, 1anti-E & 1(anti- Fy^a &anti-S)]	7.1%
Total	57	100%

A total of 57 eluates were analysed during the study period. In Table 1A demonstrates the type of antibodies present in the serum of the study population at the time of elution performed. The type of antibodies recovered in the eluate shown in the table 1B. There was a total of 20 non anti-A/B alloantibodies were recovered from 57 eluates. In which 17 antibodies were against Rh antigens, 2 were due DHTR and 1 in the group of HDFN due to other antigens. Among the 21 warm autoantibodies 6 were having Autoanti-e specificity and remaining 15 were panagglutinating autoantibodies. Anti-A/B alloantibodies were eluted from 2 out of 3 ABO HDFN neonates and 1 of this eluate was nonreactive.

Characteristics of the DAT strengths and the informative eluates in this study

Table 2 shows the strength of antecedent DATs along with the resultant informative eluates was elaborated in the above said Table 2. In total there were 50 informative eluates from 57 DAT positive cases. In which, 28 belonged to AIHA (25 wAIHA and 3 mixed AIHA), 17 belonged to Rh HDFN,3 belonged to ABO & Anti-M HDFN and 2 belonged to DHTR. In which 16 out of 57 eluates were informative for an antibody which was not detected in the serum at the time elution performed constituting 28% of the total study population. The all informative eluates found in patients having IgG antibody with and /or without the presence of other antibodies. (13 cases of AIHA, 2 cases of ABO HDFN and 1 case of DHTR). Among the 16 informative eluates, 13 eluates were causing panagglutination (recovered antibody caused agglutination of all reagent RBCs). 2 were informative eluates of ABO HDFN 1 belonged to anti-A and 1 had anti-B specificity. In the DHTR group 1 informative eluate showed the specificity of anti-Jka.

Table 2: Characteristics of the DAT strengths and the informative eluates in this study

DAT strength	Total number of elutes n=57	Total number of informative elutes n=50	No of Panagglutinating Abs/ anti-e specificity in serum n=15	No of Panagglutinating Abs in elute only	No of Rh HDFN Abs in serum	No of informative eluates in Rh HDFN	No of ABO & others HDFN Abs in Serum	No of informative eluates in ABO HDFN & others	No of DHTR Abs in serum	No of informative eluates in DHTR
1+	1	1	-	-				-	-	1 antiJk[a]
2+	13	6	1 (auto with Alloab*)	3	1	1	-	-	1 anti-c	1 anti-c
3+	17	17	2 (1 auto & 1 auto with alloab*)	4	8	8	1 (anti-M HDFN)	3 (2 ABO & anti-M HDFN)	-	-
4+	26	26	12 (anti-e 6, 4 panagg & 2 auto with alloabs*)	6	7*(maternal serum not available)	8	-	-	-	-
Total	57	50	15	13	16	17	1	3	1	2

*alloantibody was diagnosed in Autoadsorbed serum.

Glycine Acid EDTA Elution

Among 57 patients with DAT positivity Glycine Acid EDTA was carried out in 50 DAT positive patients who are diagnosed to have AIHA and HDFN. This implies that Glycine acid EDTA was not done 3 cases of CAS, 2 AIHA patients with a recent history of transfusion (< 7days) and also in 2 cases of DHTR. Glycine Acid EDTA rendered 39 samples (18 AIHA and 21 HDFN) of DAT completely negative and in the remaining 11 (AIHA)cases the strength of DAT is very much reduced than its original strength.

Type of antibodies recovered in Autoadsorbed eluates and compared with those patients' Autoadsorbed sera

5 AIHA patients with a previous history of transfusion and having increased IAT strength subjected to Autoadsorption. RBC coated autoantibodies were removed by Glycine Acid EDTA and the treated RBCs are used for Autoadsorption.

Table 4: Alloantibody in AIHA

Pt Id No	Antibody Screening in Serum	Antibody Screening After Autoadsorption	
		In Eluate	In Serum
A 4	Panagglutinins	Panagglutinins	anti-C
A 14	Panagglutinins	Panagglutinins	anti-fya,S
A 18	Panagglutinins	Panagglutinins	anti-E
A 24	Panagglutinins	Panagglutinins	anti-c
A 31	Panagglutinins	Panagglutinins	Negative

DISCUSSION

The DAT is widely used in Immunohematological laboratory test because it is simple, quick and inexpensive test. It should be performed when the presence of haemolysis has been established and is one of the most important diagnostic tests for determining Immune Hemolytic Anemias. In view of this, it is very essential to identify the patients with DAT positive and also having hemolysis to aid in the treatment and management accordingly to the specific cause of DAT positivity. With this background this study was conducted, to evaluate the DAT positive cases and to characterise them into AIHA, HDFN, HTR and/or other causes along with the various factors, antibody classes, severity and finally to perform elution studies to specify the antibody coated onto the red cells.

I. Autoimmune hemolytic anemia (AIHA) is a rare disease characterised by the production of autoantibodies against one's own red cell antigens. The degree of hemolysis depends upon the following factors such as Immunoglobulin class, subclass, titre, ability to activate complement, thermal amplitude and strength of direct antiglobulin test (DAT) [41] Accurate diagnosis of autoimmune hemolytic anemia is mandatory, as the condition requires different strategies for therapy and monitoring. Therefore awareness of demographics, clinical presentation, laboratory and serological tests is required for diagnosis of AIHAs.[6]

This present study includes 34 AIHA patients based on the features of hemolysis and Direct Antiglobulin Test positivity.

It is well known that AIHA is most common among females than males. Similar trend was noted in our study population with a male: female ratio of 1:2 (11:23) ie 67% of idiopathic AIHA patients were females. The gender distribution

among primary and secondary AIHA patients was noted to be 1:2.2 in primary AIHA and 1:1.8 for secondary AIHA.

Similarly, Allgood and Chaplin in their study reported that 60% of patients were female[48] Dacie reported 58% of patients were female.[50] Böttiger and Westerholm reported that women predominated in all age groups with the exception of the youngest (0 to 14 years), in which the sex distribution was even. In secondary WAIHA, the percentages are more varied, perhaps depending on the incidence of underlying diseases seen in referral centers. [51]

Out of 34 patients, 19 (56%) patients were diagnosed to have primary AIHA and the remaining 14 (44%) patients had secondary AIHA. This finding is in accordance to Naithani et al., they found out that frequency of primary AIHA is 65% and that of secondary AIHA is 35%.[26] In the study by Sokol et al Idiopathic/Primary AIHA accounts for 40.9% of all WAIHA.[37] Warm AIHA is the subtype that most often affects children age between 2–12 years.[39]

In this study group, the minimum age for presentation was 3 years and the maximum age was 79 years. The median age was 40 years in primary (Range 3-79years) and 26 years in secondary AIHA (Range 5-54years). Primary AIHA was most predominantly seen in >41 years of age (58%) and Secondary AIHA was more common among <45 years of age (60%).

The most common cause for secondary AIHA was Autoimmune disorders (SLE and Rheumatoid Arthritis) and followed by sickle cell anemia and lymphoproliferative disorders. This finding is reflected in the younger age of presentation of secondary AIHA.[125]

The degree of hemolysis in our study population was evaluated based on the following four parameters viz., Hemoglobin <9gm/dl, Bilirubin >2mg/dl, Lactate dehydrogenase 500IU/ml and Reticulocyte count >2%.[14,78,144]

Based on these parameters the hemolysis is classified into moderate, severe hemolysis. In our study 20(59%) patients had severe hemolysis, 13(41%) AIHA patients had moderate hemolysis. SS Das et al in their study classified hemolysis among AIHA patients into two groups only and they observed that 39.53% had severe hemolysis and 60.47% had moderate hemolysis.[14]

Correlation of strength of Polyspecific DAT with severity of hemolysis

The strength of DAT positivity correlates with the number of Immunoglobulin molecules adherent to RBC. To assess the strength of DAT various techniques have been performed viz, tube technique, CAT, flow cytometry etc. In literature many studies have been done to assess the correlation of strength of Polyspecific DAT with severity of hemolysis. In our study, polyspecific DAT, Monospecific DAT and IgG subtype was done by CAT technique.

In the present study of 34 patients with AIHA 24 patients had the DAT strength of >2+ and remaining 10 patients had <2+ DAT strength. Correlating the strength of the DAT with the severity of hemolysis, we found that Among 24 AIHA patients with >2+ DAT strength, 18 (75%) of had severe hemolysis, but only 2 (10%) out of 10 AIHA patients with <2+ DAT strength had severe hemolysis. This correlation was statistically significant with a p value of < 0.003. Similarly Wheeler et al., in their study found out that the relationship between the presences or absence of hemolysis and the DAT strength was highly statistically significant.[78] Wikman in his study revealed that there was a significant correlation between strength of DAT and severe hemolysis.[77]

Autoantibodies in AIHA

In our study we found that among the 28 AIHA patients with IgG antibodies, IgG with other antibodies or complement were found in 50% patients (ie; 17of them had IgG with combination of other autoantibodies), IgG alone was found in 32% patients (ie. Solitary IgG was found in 11 AIHA patients). Similarly in the study by Lai et al on AIHA patients he found that 55.6% of the patients had multiple autoantibodies and 44.4% had solitary IgG autoantibody.[142] In contrast to this Das SS et al,. Found out that 68.5% of the AIHA patients in his study had single autoantibody. [14]

In this conjuncture, our study found that 80% of the AIHA patients with severe hemolysis had combination of antibodies in comparison to only 15% of AIHA patients with severe hemolysis who had solitary IgG antibody. This association was statistically significant with a p value <0.002. This findings in accordance to the studies conducted by Das SS et al and Wheeler et al respectively.[14,78]

In our study C3d alone was identified in 6 (18%) of AIHA patients which raising the possibility of cold agglutination disease and 9% of the AIHA patients had mixed type AIHA having both IgG, C3d. Similarly in the study conducted by Shulman et al 8.3% of the patients having mixed type AIHA.[40]

The present study experienced that out of 34 AIHA patients 30 (88%) had autoantibodies and 4 (12%) patients had both auto and alloantibodies. Similarly, The study done by Wikman A et al had 28% of alloantibodies in AIHA patients all of them fell in moderate hemolysis group.[77] Similarly the study conducted by Branch DR and Petz LD 25-47% of sera from AIHA patients showed the presence of alloantibodies.[141]

IgG subtypes

IgG1 was the most common subtype present in 11 (40%) patients, followed by IgG1 and IgG3 which was present in 8 (29%) patients and solitary IgG3 was found in 4 (14%) patients with AIHA. In our study 5 (17%) patients had neither IgG1 nor IgG3. In the study conducted by Das SS et al., 46.5% of AIHA patients, the subclass was IgG1 or IgG3 or both, while in the remaining patients, no IgG1 or IgG3 was detected.[14] In the other study, IgG1 was the most frequent subclass (96%) coating the red cells.[29]

The presence of both IgG1 and IgG3 has a significant effect on severity of hemolysis. A total of 8 patients were noted to have both IgG1 and IgG3 in which 7 (87%) had severe hemolysis in par with 1 (13%) moderate hemolysis. A similar impact was noted in patients with solitary IgG1 [95% CI= 25.0570 to 88.9932; P = 0.0042]. Among 11 AIHA patients with only IgG1 autoantibody 10 (90%) had severe hemolysis and 1 (10%) 95% [CI= 39.2760 to 91.4404; P = 0.0002] was having moderate hemolysis. Both of these associations were statistically significant. Among 4 AIHA patients who had IgG3 subclass, 3(75%) had moderate hemolysis and 1 (25%) had severe hemolysis. In 5 AIHA patients who had neither IgG1 nor IgG3, 4 (80%) had moderate hemolysis and 1 (10%) had severe hemolysis.

Similarly in Das SS et al study 60% of the patients with combination of IgG1 with IgG3 and IgG1 alone were more likely to present with severe hemolysis as compared to 21.7% of the patients having IgG3 only and neither IgG1 nor IgG3.[14]

THERMAL AMPLITUDE

Out of 34 AIHA patients, 25(74%) warm autoantibodies reacted at 37˚C only, among 6 (18%) Cold AIHA in which 3 (9%) had thermal amplitude of 4˚C to 22˚C and 3 (9%) had wide thermal amplitude of 4˚C to 37˚C. The remaining 3

patients belonged to Mixed type AIHA. In a similar type of study conducted by Das SS 81.4% was WAIHA, 16.3% were mixed type and there was only one cold AIHA (2.32%). [14]

ELUTION (by Glycine Acid and Glycine Acid EDTA**)**

Among the 34 DAT positive samples, Elution was done in all AIHA patients using commercially available kits, 28 elutes were reactive and 6 elutes were nonreactive. Among 28 reactive eluates 22 were showing panagglutination, in which 21 (73%) were WAIHA with, 1 (4%) was due to mixed AIHA and 6 (23%) AIHA samples showed the presence of autoantibody specificity as anti-e which was confirmed with serum studies (4 were due to WAIHA and 2 were mixed AIHA).Similar to this a study conducted by Marilyn Johnston FM, Mary Kay Belota, observed that 68% of patients, in which 37% yielded positive eluates and 63%, had nonreactive ones. Of the positive elution studies, 73% demonstrated only warm autoantibody on red blood cells. 2.5% of these had warm autoantibody in serum as well. 3% of these specimens were from previously but not recently transfused patients and had alloantibody/ies in serum. 33% eluates with positive results were from patients transfused 26-48 days before testing.[97] Similarly Dacie JV in his study reported that Autoanti-e was the most common specificity; it has been pointed out that the reported relative incidence of different specific Rh autoantibodies corresponds well with the incidence of Rh antigens in the population (i.e., ‘e’ antigen is present on the RBCs of approximately 98% of the population).[39]

Among 34 patients with AIHA Glycine Acid EDTA was carried out in patients who are diagnosed case of AIHA and in patients with h/o previous transfusion more than 3 months. Glycine Acid EDTA rendered DAT completely negative in 18 of the cases and in remaining 11 cases the strength of DAT is reduced than its original strength. Similarly in the study conducted by Katharia R Glycine

Acid EDTA 59 DAT positive samples showed decrease in reaction of which 22 samples were DAT negative.[8]

5 WAIHA patients who were having IAT ≥ DAT further submitted to Autoadsorption to identify the presence of any Alloantibodies. Out of 5 patients all RBC coated autoantibodies were removed by Glycine Acid EDTA and the treated RBCs are used for Autoadsorption. The Autoadsorption revealed the presence of clinically significant alloantibodies in 4 out of 5 patients and one patient had no alloantibodies. Adsorptions with autologous RBCs, Laine and Beattie,[146] James and colleagues,[147] Issitt et al,[148] and Morel and coworkers[149] detected alloantibodies in 27%, 32% , 38%, and 40% of sera, respectively.

Autoantibody specificity

In 19 of 34 (55%) autoantibodies reacting with all test erythrocytes (panagglutination) were detected, Autoantibodies with specificity against Rh antigen (anti-e) were identified in 5 of 20 patients in the group with severe haemolysis, in 1 of 13 with moderate haemolysis and Autoantibodies with specificity against I antigen (anti-I) were identified in 1 of 20 patients in the group with severe haemolysis, in 5 of 13 with moderate haemolysis. Jenkins and coworkers found that sera containing cold autoagglutinins that had previously been called “nonspecific cold agglutinins” had anti-I specificity and it is more common to other cold autoantibodies.[100]

Auto and Alloantibody in AIHA vs Severity of hemolysis

Alloantibodies were identified in 4/20 (20%) in relation to AIHA patients with severe hemolysis and all of them had previous history of transfusion. 3 of 4 patients had single clinically significant alloantibody and 1 of 4 had multiple clinically significant alloantibodies. The association between transfusion and

alloantibody formation is statistically significant with a p value of 0.000. The study done by Wikman A et al had 28% of alloantibodies in AIHA patients all of them fell in moderate hemolysis group.[77] Similarly the study conducted by Branch DR and Petz LD 25-47% of sera from AIHA patients showed the presence of alloantibodies.[141]

Hemolytic Diseases of Fetus and New-born (HDFN)

DAT positivity in new-born is only due to HDFN either due to Rh incompatibility or due to ABO incompatibility between mother and fetus.

Etiology

In our present study, Out of 21 cases of DAT positive HDFN cases received 17 (81%) were due to Rh, 3 (14%) were due to ABO HDFN and 1(5%) is due to others. Similarly in the study conducted by Dharmesh Chandra Sharma et al Rh incompatibility was the commonest cause of HDN with 61 (55.5%) cases of RhD HDN whereas ABO and other group HDN cases were 30 (27.3%) and 19 (17.3%) respectively.[67]

Correlation of Hyperbilirubinemia (TSB >15mg/dl) with HDFN

From a total of 21neonates, 16 (76%) with hyperbilirubinemia had TSB >15mg/dl, in which 5 infants needed ET and 16 were treated with phototherapy. The remaining 5 Infants who had mild hyperbilirubinemia TSB < 15mg/dl needed no intervention. Similarly in the study done by Swathi Chacham et al 41/48(85%) had hyperbilirubinemia with TSB >15mg/dl all of them needed Phototherapy and/or ET.[155]

There is statistically significant correlation between hyperbilirubinemia with the management of HDFN and has a p value of < 0.000. (Mann-Whitney test / Two-tailed test)

Mean HGB value in Moderate to Severe HDFN and mild HDFN patients

Mean hemoglobin was assessed in patients with moderate to severe HDFN and mild HDFN by unpaired T test. The mean hemoglobin of moderate to severe hemolysis is 13.46gm/dl and of mild HDFN is 16.04gm/dl. Similarly Mollison et al,. stats that Moderately severe HDFN had a cord Hb concentration of 12.8 g/dl, and approximately 50% of the HDFN neonates had cord Hb concentrations of 14.5 g/dl or more, 30% had cord Hb values between 10.5 and 14.4 g/dl, and about 20% had Hb values of between 3.4 and 10.4 g/dl. The probability of survival diminishes as the cord Hb concentration falls. A level of 4 mg/dL or more of cord blood bilirubin (normal range, 0.7–3 mg/dL) or a rapidly rising bilirubin is suggested as an indication for exchange transfusion. If the cord total bilirubin is less than 4–5 mg/dL and rising only slowly, phototherapy might suffice to correct the problem.[109]

There is statistically significant difference in mean hemoglobin in patients with severe and moderate hemolysis (p <0.0013).

Transplacental passage of maternal IgG results in antibody coating of the fetal red cells. High alloantibody concentrations lead to a strong DAT, whereas low concentrations or binding affinity result in a weak or, by traditional methods, often negative DAT. A strong positive DAT is observed particularly with anti-D; however, the reaction is more variable with alloantibodies against other Rh antigens and antigens such as Kell, Duffy, Kidd, and MNSs, which are well expressed on fetal red cells.[5]

Correlation between Strength of DAT with severity of HDFN

Out of 16 neonates with >2+ DAT strength 15 neonates had Moderate to severe HDFN and 1 neonates had mild HDFN and among 5 neonates having <2+ as strength of DAT, 4 of them presented with mild HDFN and1 neonate was having Moderate to severe HDFN. A strong positive DAT is observed particularly with anti-D; however, the reaction is more variable with alloantibodies against other Rh antigens and antigens such as Kell, Duffy, Kidd, and MNSs, which are well expressed on fetal red cells.[5]

Correlation between Strength of DAT with severity of Rh HDFN

Out of 17 neonates with Rh HDFN 15 neonates with moderate to severe hemolysis had >2+ DAT strength and 2 neonates had <2+ as strength of DAT and presented with mild HDFN. There is significant correlation between Rh HDFN and severity of HDFN with a p value of 0.012.Similarly in a letter to editor written by Mustafa Aydin et al, they stated that severity of Rh HDFN is well correlated with strength of DAT.[151]

Specificity of DAT in HDFN

Out of 21 cases of HDFN (100%) all of them had anti-IgG only in Monospecific DAT. Similarly in the study conducted by Pollock & Bowman only IgG was the only antibody identified in cases of HDFN.[30]

IgG subtype of DAT in HDFN

Out of 21 HDFN cases, IgG1 was identified in (11) 52% of the neonates; IgG1 & IgG3 (6) were found in 29% of the neonates, IgG3 was found in (3)14% of the neonates and neither IgG1 nor IgG3 were seen in 5% (1) neonate. Similarly in a study done by Frankowska and Gorska, they found that 87.6% had IgG1 Rh

antibodies, 23% contained IgG2 antibodies, 56.9% contained IgG3 antibodies, and 7.7% contained IgG4 antibodies.[31]

Correlation between IgG subclass and severity of HDFN

Out of 17 Rh HDFN 2 neonates had mild HDFN and 15 neonates had Moderate to severe HDFN. The distribution of IgG1, IgG1& IgG3 and IgG3 was 11 (65%), 3 (14%) and 3 (14%) respectively among the neonates with Rh HDFN. This present study has statistical significance between the subclass and severity of Rh HDFN. Similarly, Nance et al., in their study found that severe HDN was associated with IgGl antibodies more frequently than with IgG3 antibodies.[144]

Glycine Acid Elution

Among the 21 neonates acid elution was done in all the infants. 20(95%) eluates were reactive and 1(5%) eluate of ABO HDFN was nonreactive.

In this study there is a statistically significant correlation between the DAT positive neonates and antibody identification in the eluate with a p value of <0.0001. R.H. Finck et al., in their study found that antibodies eluted from cord blood RBCs was 100% (7 of 7). He also mentioned that apart from ABO HDFN all other antibody mediated HDFN can be diagnosed antenatally. However, Elution can be of useful value in diagnosing clinically significant ABO HDFN and difficulty in obtaining maternal serum for other HDFN.[96]

Glycine Acid EDTA Elution in HDFN

Out of 21 HDFN samples Glycine Acid EDTA elution was done in all the samples and DAT was rendered negative. Rh phenotyping along with other specific phenotypes were identified in the above treated RBCs. However kell antigens (K,k) were destroyed by this elution method. Rahul Katharia et al in his study found that

the best method for obtaining antibody free DAT positive RBCs is Glycine Acid EDTA.[8]

Antibodies found in neonates with HDFN by elution

Out of 17 Rh HDFN neonates Anti-D was found in 13 (76%) neonates, anti-D & anti-C was found in 2 (12%) neonates, anti-c was found in 2 (12%) neonates only. Among 3 ABO HDFN Anti-A was found in 1 Neonate and Anti-B was found in 1 neonate and among other blood group antigens anti-M was found in 1 neonate. Huub H.vanRossum et al., study explains the high sensitivity of both techniques (DAT and Elution) on detecting neonatal erythrocytes sensitized with anti-A and anti-B as the maternal serum can be used to detect the HDFN due to other blood group antigens.[77] R.H. Finck et al., stated in his study that elution can be of useful value in diagnosing clinically significant ABO HDFN and difficulty in obtaining maternal serum for other HDFN.[95]

DELAYED HEMOLYTIC TRANSFUSION REACTIONS (DHTRs):

A transfusion reaction can be classified as an DHTR only if evidence of hemolysis (i.e., reduced red cell survival) is documented.

III. a. The strength of polyspecific DAT in DHTR

Out of 2 cases 1 had 3+ in polyspecific DAT and 1 had 1+ reaction in polyspecific DAT.

III. b. Features of Hemolysis

	HGB gm/dl	**TSB mg/dl**	**LDH IU/ml**	**Retic**
Patient 1	7.3	2.8	615	4%
Patient 2	8.8	2.4	532	2%

Pineda et al. in his study found out that the most constant features of DHTRs are fever and a fall in Hb concentration. Other features that are often observed are jaundice and rarely haemoglobinuria.[104]

Erwin Strobel In his study describes that in case of a DHTR, rise in bilirubin, reduction of haptoglobin, and sometimes slightly elevated free hemoglobin in the patient's plasma can be found. In some cases, hemoglobinuria may also occur. The hemoglobin value of the patient will drop again about 1–2 weeks after blood transfusion without bleeding or other known reasons. The reticulocyte count will rise if the patient's disease allows this reaction, and in the blood smear spherocytes can be seen.[145]

III. c. Distribution of antibodies by monospecific DAT

Out of 2 cases of delayed hemolytic transfusion reactions both of them had IgG and C3d in their coated RBCs identified by monospecific DAT. Salama and Muller-Eckhardt [35] in their study found that all the patients were found to have both IgG and C3d antibodies detected on their RBCs after the delayed hemolytic reaction. RBC bound C3d is detectable by DAT for weeks and even months after the transfusion. Similarly O Nathalang el at., in their study found that most of the patients who had IgG antibodies or IgG and C3d coated on their RBC (91.6%) had a history of blood transfusions.[154]

III. d. Distribution of IgG subtypes

Both patients of delayed hemolytic transfusion reactions had IgG1 subclass with 1:1 dilution which was identified by IgG subclass card. IgM, IgG3, and IgG1 are able to start the classical pathway of complement activation, IgG3 and IgG1 also can initiate extravascular RBC clearance without complement activation.[36, 82]

IV. e. Elution in DHTR

In Glycine Acid elution of both DAT positive DHTR samples we found the presence of Anti-Jk^{a} and anti-c both are clinically significant antibodies. However 1 antibody (Anti-c) was found in serum of the patient. Vamvakas et al in their study found that the prevalence of DHTR was higher when the implicated RBC alloantibody was anti-Jk^{a} with a p value of <0.000.[157] In the study conducted by Ness et al Alloantibody was eluted from the RBCs of all patients.[36] Hoeltge and coworkers studied alloimmunization and found the most common specificities were anti- K (23%), anti-E (18%), anti-D (12%), anti-Lea (7.3%), anti-C (6.3%), anti-Fya (5.7%), anti-c (4.4%), and anti-Jka (3%).[157]

Glycine Acid EDTA Elution

Glycine Acid EDTA Elution was not performed in both the DHTR patients since they had h/o transfusion 6 days and 10 days back respectively.

Elution of DAT positive samples in overall view

A total of 57 eluates were analysed during the study period. There was a total of 20(35%) non anti-A/B alloantibodies (mainly among HDFN and DHTR cases) were recovered from 57 eluates. Among the 24 (42%) warm autoantibodies [6 (11%) were having Autoanti-e specificity and remaining 18 (32%) were panagglutinating autoantibodies]. Anti-A/B alloantibodies were eluted from 2 (4%) out of 3 ABO HDFN neonates. 4 (7%) of WAIHA patients had both auto and alloantibodies. Similarly in the study done by Mark Yazer there was 12.4% nonanti-A/B alloantibodies were recovered; 43.8% warm autoantibodies and 1.5% had both auto and alloantibodies.[153] In our study 16 were informative eluates in which serum antibody detection was negative. However, among these only 3 eluates had antibody specificity (2 anti A/B and anti Jka) remaining 13 were panagglutinins. In a study

done by R.H. Finck he found that in HDFN out of 7 cases informative eluates were found in 5 cases all of them belonged to ABO HDFN remaining 2 belonged to Rh HDFN and they also present in maternal serum. [96]

Overall rate of informative eluates in our study was 5.3% excluding the panagglutinins and 1.9% of these informative eluates were due to DHTR. Similarly, in the study done by Mark Yazer Overall rate of informative eluates was 12.7%. Further, 1.7% of these informative eluates were due to DHTR. [153] Similarly Johnston and Belota found only two (2.4%) eluates that revealed a new alloantibody in the eluate which was not in the serum. Both of these patients suffered a delayed hemolytic reaction.[97]

Glycine Acid EDTA Elution

Among 57 patients with DAT positivity Glycine Acid EDTA was carried out in 50 DAT positive patients who are diagnosed to have AIHA and HDFN. This implies that Glycine acid EDTA was not done 3 cases of CAS, 2 AIHA patients with a recent history of transfusion (< 7days) and also in 2 cases of DHTR. Glycine Acid EDTA rendered 39 samples (18 AIHA and 21 HDFN) of DAT completely negative and in the remaining 11 (AIHA) cases the strength of DAT is very much reduced than its original strength. In the study done by Rahul Katharia et al., they found that among 93 DAT positive samples, 59 samples showed decreased strength and 22 were DAT negative.[8]

SUMMARY

The present study on evaluation of DAT positive cases by elution study is done by assessing serological characterization of red cell bound antibodies with regard to antibody class, subclass, DAT strength and their correlation with in vivo haemolysis. The specificity of the auto and alloantibodies was identified by using eluate prepared from acid elution technique. The presence of additional alloantibody is identified after auto-adsorption with Glycine Acid EDTA treated cells. Further, to confirm the specificity of the antibodies, the red cells (treated with Glycine Acid EDTA) were phenotyped for the corresponding antigens,

- Our study included total number of 57 DAT positive cases.
- Among 57 DAT positive cases 34 were AIHA, 21 were HDFN and 2 were DHTR.
- In 34 AIHA patients, 25 were WAIHA, 3 belonged to Mixed AIHA and 6 were due to Cold AIHA.
    - In general, the gender distribution among AIHA patients was (male: female ratio) 1:2.
    - 20 (56%) patients were diagnosed as Primary AIHA and 14 (44%) patients as Secondary AIHA.
    - The most common cause for secondary AIHA was due to Systemic Autoimmune disorders [SLE (n=4) & Rheumatoid Arthritis (n=1)].
    - Other cases were associated with Sickle Cell Anaemia (n=2), Lymphoproliferative Disorders (n=3), Pure Red Cell Aplasia (n=1), Ovarian Tumours (n=2), HIV (n=1), and Carcinoma Cervix (n=1).
    - Out of 24 cases with more than 2+ DAT strength, 18 showed severe hemolysis.
    - 17 cases (50%) showed IgG class of Immunoglobulins along with IgM, IgA and /or Complements.

- 11 cases (32%) showed exclusively IgG class of Immunoglobulins.
- 6 cases (18%) showed only the presence of Complements.
- Out of 20 cases of severe hemolysis, 16 had combinations of various classes of Immunoglobulins along with Complements (IgG & C3d 11, IgG & IgA 2, IgG, IgM & C3d 1and IgG, IgM, IgA &C3d 2).
- Out of 28 cases with the presence of IgG Immunoglobulins (IgG alone 11,IgG & C3d 12, IgG & IgA 2, IgG, IgM & C3d 1and IgG, IgM, IgA &C3d 2).
- Out of 28 cases with IgG Immunoglobulins, 11 had IgG 1 subclass, 8 had IgG1 & IgG3, 4 cases had IgG3. 5 cases belonged to neither IgG1 nor IgG3.
- Out of 6 cases with the presence of only complements, 3 showed reaction at 4 ^{o}C to 37 ^{o}C in which 1 (17%) showed severe hemolysis with titre of 2048.
- Out of 34 cases, 30 (88%) AIHA patients had autoantibodies and 4 (12%) patients had both auto and alloantibodies, these 4 cases had previous history of transfusion. The alloantibodies identified were Anti-C (1), Anti-E (1), anti-c (1) and anti Fya along with anti-S in (1) patient.
- Out of 30 cases with only autoantibodies, 6 cases WAIHA (n=-4) and Mixed AIHA (n=2) showed anti-e specificity, 6 cases of cold AIHA showed anti-I specificity, the remaining 18 cases showed panagglutination.

➢ In 21 DAT positive cases of HDFN 17 (81%) were due to Rh HDFN, 3 (14%) were due to ABO HDFN and 1(5%) case was due to anti-M antibodies.

- Out of 17 neonates with Rh HDFN 15 neonates with moderate to severe hemolysis had >2+ DAT strength and 2 neonates had <2+ as strength of DAT and presented with mild HDFN.

- All the 21 DAT positive HDFN neonates had only IgG antibody in their RBCs, including 3 cases of ABO HDFN.
- Out 21 HDFN neonates, IgG1 was identified in (11) 52% of the neonates; IgG1 & IgG3 (6) were found in 29% of the neonates, IgG3 was found in (3)14% of the neonates and neither IgG1 nor IgG3 were seen in 5% (1) neonate.
- All 15 neonates with moderate to severe hemolysis had IgG1 subclass or IgG1 along with IgG3 subclass of Immunoglobulins.
- The neonate with neither IgG1 nor IgG3 showed anti-M antibody.
- Out of 21 eluates 20 were informative with presence of alloantibodies. The nonreactive eluate prepared from the remaining one neonate belonged to ABO HDFN, which showed anti-A specificity on cold elution.
- Out of 20 eluates 16 were compared and confirmed with maternal serum.

➢ Among 2 cases of DHTR, 1 had "anti-Jka" and the other had "anti-c" antibody.

- Monospecific DAT on coated RBCs from these 2 cases revealed presence of both IgG class of Immunoglobulins and C3d.
- Both patients of delayed hemolytic transfusion reactions had IgG1 subclass.
- Both the patients had history of previous transfusion and showed features of hemolysis.
- Out of these 2, anti-Jka was identified in the eluate only and anti-c was identified in both serum and eluate.

➢ The RBCs treated with Glycine Acid EDTA from the total of 57 cases, 27 cases (6 cases of AIHA and 21 cases of HDFN) showed the presence of corresponding antigen.

CONCLUSION

In our study on evaluation of DAT positive cases by elution study revealed a strong association between DAT strength and the severity of hemolysis. Further, we observed that the presence of IgG immunoglobulins in combination with IgM, IgA and complements elicit severe hemolysis in AIHA cases in comparison to exclusive presence of only IgG. However, in cases of HDFN the pathogenesis of hemolysis was exclusively due to the presence of IgG.

Our study included two cases of DHTR, one was due to anti-c and another was due to anti-Jka, in both these cases we found these antibodies along with complements. In all cases of DAT positive hemolysis with the presence of IgG immunoglobulins irrespective any aetiology, the most common subclass identified was IgG 1 and IgG 3.

In six of cold AIHA cases in our study, one case had shown severe hemolysis due to the presence of high-titre (2048) anti-I antibody along with complements.

The specificity of auto and alloantibodies were identified by adsorption and elution techniques, which revealed exclusively anti-e in 6 cases of WAIHA and 4 cases with previous history of transfusion had panagglutinating autoantibodies along with the presence of clinically significant alloantibodies.

Our study suggests a significant association between the strength of DAT, the IgG class and subclass (IgG 1 & IgG 3) of Immunoglobulins either alone or in combination with other classes of immunoglobulins and/or complements.

The Glycine Acid EDTA elution in DAT positive immune haemolytic anemia cases provide uncoated RBCs for exact phenotyping and autoadsorbed serum for cross match compatibility. This study further reiterates the importance of acid elution in DAT positive of HDFN and DHTR cases in identification of the specific alloantibody.

Further, this study reinstates a schematic approach in dealing with DAT positive immune haemolytic anemia cases by designing institutional based algorithm for efficient patient management.

LIMITATIONS OF THE STUDY

1. This study does not represent the true prevalence of AIHA, HDFN and DHTR since MMC and Department of Transfusion Medicine, The TN Dr.M.G.R Medical University are referral centres.

2. Criteria for assessing severity of haemolysis was derived from a previous publication from Wheeler et al, Das SS et al and Mishra JP et al as no clinical guidelines available for the same.

3. Patients recruited for the study belonged to different time points in the course of their disease.

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PLAGIARISM CERTIFICATE - II

This is to certify that this dissertation work titled EVALUATION OF DIRECT ANTIGLOBULIN TEST POSITIVE CASES BY ELUTION STUDY of the candidate **Dr.G.KAVITHA** with registration Number for the award of **M.D (IH &BT)** in the branch of **M.D BRANCH – XXI (Immunohaematology &Blood Transfusion)** I personally verified the urkund.com website for the purpose of plagiarism Check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **1 percentage** of plagiarism in the dissertation.

Guide & Supervisor sign with Seal

PARTICIPANT CONSENT FORM

Participant's name: Address:

EVALUATION OF DIRECT ANTIGLOBULIN TEST POSITIVE CASES

BY ELUTION STUDIES

The details of the study have been provided to me in writing and explained to me in my own language. I confirm that I have understood the above study and had the opportunity to ask questions. I also understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without the medical care that will normally be provided by the hospital being affected. I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s). I have been given an information sheet giving details of the study. I fully give my consent to participate in the above study.

Signature of the Participant: Date:

Signature of the Witness: Date:

Signature of the Investigator: Date:

CONSENT FORM (for participants less than 18 years of age)

Participant's name: Address:

Parent/LAR's name:

EVALUATION OF DIRECT ANTIGLOBULIN TEST POSITIVE CASES

BY ELUTION STUDIES

The details of the study have been provided to me in writing and explained to me in my own language. I confirm that I have understood the above study and had the opportunity to ask questions. I also understand that my child/ward's participation in the study is voluntary and that I am free to withdraw my child/ward at any time, without giving any reason, without the medical care that will normally be provided by the hospital being affected. I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s). I have been given an information sheet giving details of the study. I fully give consent for the participation of my child/ward in the above study.

Assent of child/ward(for participant's 7 to 18 years of age)

Signature of the Parent/LAR: Date:

Signature of the Witness: Date:

Signature of the Investigator: Date:

PATIENT INFORMATION SHEET

TITLE OF THE STUDY

Elution studies in DAT positive cases

NAME OF THE INVESTIGATOR

Dr.G.Kavitha, 2ND Year P.G, MD (IH & BT)
The TN Dr.M.G.R Medical University,
Guindy, Chennai-32

NAME OF THE GUIDE:

Dr.Swathandran Hamsavardhini, MD(IH&BT),
Associate Professor,
Department of Transfusion Medicine,
The Tamilnadu Dr.M.G.R. Medical University,
Chennai-32.

PURPOSE OF THE STUDY

The direct antiglobulin test is used most commonly to investigate possible haemolytic transfusion reactions, haemolytic disease of the fetus and newborn (HDFN), autoimmune haemolytic anaemia and drug induced immune haemolysis. Elution removes antibody molecules from the red cell membrane either by disrupting the antigen or changing conditions to favor dissociation of antibody from antigen. Many techniques are available, and no single method is best in all situations. If an eluate prepared by one technique is unsatisfactory, it may be helpful to prepare another eluate utilizing a different technique.

PROCEDURE:

After getting consent from the patient, complete details regarding the patient which includes name, age, gender, IP number, clinical diagnosis of the patient will be obtained from the questionnaire by the principal investigator. 5ml of patient's blood is collected in EDTA tube. Blood grouping and typing, DAT, IAT and AutoControl, Antibody screening and Identification and Elution will be performed at the The Department of Transfusion Medicine The Tamilnadu

Dr.M.G.R. Medical University, Guindy, Chennai. Acid alters charge of proteins causing a change in structural configuration. Most allo and autoantibodies are detected by acid elution technique.

DURATION

August 2017 to September 2018.

BENEFITS AND RISKS

We can differentiate between auto and allo antibody. Based on which treatment can be changed and antigen matched blood can be transfused which will save the life of the patient.

There will be less than minimal risk for patients enrolled as it is a continuous process of routine investigation.

CONFIDENTIALITY

Your privacy will be protected as permitted by law. Only your researcher and ethical committee members will have access to data collected during the study.

PARTICIPATION

Your participation in this study is voluntary and you are free to decide now are later whether to continue or discontinue from the study any time without giving any reason, the medical care to you will be normally provided by the hospital. Your data or results that are generated from this study will be used or shared only for scientific purpose(s).

I. Investigator:

Dr.G.Kavitha ,
MD(IH&BT),Post Graduate student,
The TN Dr.M.G.R medical University,
Guindy, Chennai.

II. Guide:

Dr.Swathandran Hamsavardhini, MD(IH&BT),
Associate Professor,
Department of Transfusion Medicine,
The Tamilnadu Dr.M.G.R. Medical University,
Chennai-32.

III. Co-Guide:

DR.Margret,MD.,DM.,
Professor and Head,
Department of Hematology,
Rajiv Gandhi General Hospital,
Chennai.

NAME OF THE PATIENT :

SIGNATURE :

DATE :

Signature of the Investigator: Date:

PROFORMA FOR DAT POSITIVE CASES

Patient's Name: Hospital Number:

Address:

Patient's ABO/D typing: Patient's DAT :

H/o Previous transfusion :

H/o pregnancy:

H/o associated illness:

H/o Transplantation:

Drug history :

Hematology & Biochemical Markers of Hemolysis:

Hb (gm/dl) :

Reticulocyte count :

S.bilirubin (mg/dl) :

LDH (IU/ml) :

Serological Evaluation:

DAT Strength :

Polyspecific card :

Monospecific card :

IgG Subclass :

Elution Procedure:

(Acid elution) :

Antibody Screening and Identification;

	Reactions with eluate	Reactions with Last Wash Control
3 cell panel		
11 cell panel		

REACTIVE :

NON-REACTIVE :

Glycine Acid EDTA Elution :

DAT Strength :

NEGATIVE :

Decreased :

Interpretation of elution results:

Date:

நோயாளியின் ஒப்புதல் படிவம்

ஆராய்ச்சியாளர் என்னிடம் இந்த ஆய்வானது இரத்த சிவப்பு அணுக்களுக்கு எதிராக உண்டாகும் ஆண்டிபாடிகளினால் ஏற்படும் இரத்தசோகைக்கானது என்று எடுத்துக் கூறினார். இதற்காக சுமார் 5மி.லி இரத்தம் என்னிடமிருந்து எடுத்து பரிசோதனை செய்யப்படும் என்று விளக்கினார். நான் இந்த ஆய்வில் பங்குபெற முழுமனதுடன் சம்மதிக்கிறேன். மேலும் கூடுதல் பரிசோதனை இந்த ஆய்வுக்காக தேவைப்படுமேயானால் அதற்கும் முழுமனதுடன் சம்மதிக்கிறேன். இதனால் எனது சிகிச்சை எந்த விதத்திலும் பாதிக்காது என்பதனையும், இந்த ஆய்விலிருந்து எந்த நேரத்திலும் விலக எனக்கு உரிமை உள்ளது எனவும் ஆராய்ச்சியாளர் விளக்கினார்.

நோயாளியின் பெயர்:

கையொப்பம்:

தேதி:

பங்கேற்பாளர்களுக்கான தகவல் படிவம்

இரத்தமழிவுச் சோகையில் இரத்த சிவப்பு அணுக்களுக்கு எதிராக ஏற்படும் ஆண்டிபாடிகளின் தன்மை குறித்து கண்டறியும் ஆய்வு.

குறிக்கோள்:

சாத்தியமான தன்னுடல் தோற்றமுள்ள ஆண்டிபாடிகளி னால் ஏற்படும் இரத்தமழிவு சோகை, மருந்து தூண்டப்பட்ட நோய் எதிர்ப்பு ஆண்டிபாடிகளினால் ஏற்படும் இரத்தமழிவுச் சோகை கரு மற்றும் பிறப்பு (ஹெச்ஷஎஃப்என்) இரத்தமழிவுச் சோகையில், இரத்த சிவப்பு அணுக்களுக்கு எதிராக ஏற்படும் ஆண்டிபாடிகளின் தன்மை குறித்து கண்டறிய ஆய்வு மேற்கொள்ளப் படும். ஆய்வு செய்ய நேரடியாக எல்யூஷன் சோதனை பயன்படுத்தப்படுகிறது. சிவப்பு செல் சவ்வு இருந்து ஆன்டிபாடி மூலக்கூறுகளை நீக்குதல் அல்லது ஆன்டிஜெனின் இருந்து ஆன்டிபாடி விலகல் ஆதரவாக மாற்ற நிலைகள் மாற்றுவதன் மூலம். பல நுட்பங்கள் கிடைக்கின்றன, எல்லா சூழ்நிலைகளிலும் எந்த ஒரு எல்யூஷன் முறையும் சிறந்ததல்ல. ஒரு தொழில் நுட்பத்தால் தயாரிக்கப்படும் ஒரு தூண்டுதல் திருப்தியற்றதாக இருந்தால், வேறு நுட்பத்தை உபயோகிப்பதற்கான மற்றொரு உதவியை தயாரிக்க உதவியாக இருக்கும்.

செய்முறை:

நோயாளியின் அனுமதியின்போது, நோயாளியின் பெயர், வயது, பாலினம், ஐபி எண், நோயாளிக்கு மருத்துவ நோயறிதல் ஆகியவை அடங்கும் நோயாளிக்கு முழுமையான விவரங்கள் முதன்மை புலன்விசாரணையாளரால் கேள்வி கேட்கப்படும்.

நோயாளியின் இரத்தம் 5ml EDTA குழாயில் சேகரிக்கப்படுகிறது. இரத்த பிரிவு மற்றும் தட்டச்சு, DAT, IAT மற்றும் AutoControl, ஆன்டிபாடி ஸ்கிரீனிங் மற்றும் அடையாளம் காணல் மற்றும் எல்யூஷன் இரத்த பரிசோதனை டாக்டர் எம்.ஜி.ஆர் மருத்துவ பல்கலைகழகத்திலலுள்ள குருதியேற்று துறையில் ஆய்வுக்கு உட்படுத்தப்படும்.

படிப்பு காலம்:

ஆகஸ்ட் 2017 முதல் செப்டம்பர் 2018 வரை

பலன்கள்:

இரத்தமழிவுச் சோகையில் இரத்த சிவப்பு அணுக்களுக்கு எதிராக ஏற்படும் ஆண்டிபாடிகளின் தன்மை குறித்து கண்டறிய இந்த ஆய்வு உதவும். மேலும் நோயாளிகளின் இரத்தவகையை கண்டறிந்து அவர்களுக்கு சரியான சிகிச்சை அளிக்க உதவும்.

பாதிப்புகள்:

இது வழக்கமான பரிசோதனைகளின் தொடர்ச்சியான செயல்முறை என்பதால் பதிவுசெய்யப்பட்ட நோயாளிகளுக்கு இடர் குறைவாக இருக்கும்.

இரகசியபாதுகாப்பு:

உங்கள் மருத்துவ தகவல் (தனிப்பட்ட விவரங்கள், உடல் பரிசோதனை, விசாரணை, மற்றும் உங்கள் மருத்துவ வரலாறு) பற்றிய தனியுரிமை தொடர்பாக இரகசியத்திற்கு உரிமை உள்ளது. இந்த ஆவணத்தில் கையெழுத்திடுவதன் மூலம், உங்கள் தரவை பார்வையிட, ஆராய்ச்சிக் குழு விசாரணை, மற்ற ஆய்வுப் பணியாளர்கள் மற்றும் நிறுவன நெறிமுறைகள் குழு ஆகியவற்றை அனுமதிக்க வேண்டும். விஞ்ஞான

சஞ்சிகைகளில் வெளியிடப்பட அல்லது விஞ்ஞானக் கூட்டங்களில் பங்கேற்க அனுமதி வழங்கப்பட்டிருந்தால், இந்த ஆய்வின் தகவல்கள் உங்கள் அடையாளத்தை எந்தவிதத்திலும் வெளிப்படுத்தாது.

பங்களிப்பு:

இந்த ஆராய்ச்சியில் தங்களின் பங்கேற்பு முற்றிலும் தன்னார்வமாக உள்ளது. எந்தவொரு காரணமும் இன்றி இந்த ஆய்வில் எந்த நேரத்திலும் ஆய்வுக்குப்பின் எந்த நேரத்திலும் திரும்பப் பெற உங்களுக்குஉரிமை உள்ளது. இருப்பினும், நீங்கள் சிகிச்சையை நிறுத்துவதற்கு முன்னர் ஆராய்ச்சி குழுவிடம் பேசுதல் / செயல்முறைகளை நிறுத்துதல் போன்றவற்றைப் பரிந்துரைப்பது நல்லது. இந்த ஆராய்ச்சியில் பங்கேற்க வேண்டாம் என்ற உங்களின் முடிவினால்,தங்களுக்குமருத்துவ ர்களின் பராமரிப்பு அல்லது உங்களின் மருத்துவ சிகிச்சைஅல்லது மருத்துவமனையுடன் உங்கள் உறவு பாதிக்காது. நீங்கள் கவனித்துக் கொள்ளப்படுவீர்கள், உங்களுக்குரிய எந்த நன்மையையும் நீங்கள் இழக்க மாட்டீர்கள். இந்த ஆய்விற்கான சிகிச்சை மற்றும் ஆராய்ச்சியின் செலவிற்கான பணம் உங்களிடமிருந்து சேகரிக்கப்படாது.

நோயாளியின் பெயர்:

கையொப்பம்:

இடம்:

தேதி:

I. ஆய்வாளர்

மரு.கோ.கவிதா

மருத்துவ முதுகலை பட்டப்படிப்பு மாணவி,

தமிழ்நாடு டாக்டர் எம்.ஜி.ஆர் மருத்துவ பல்கலைக்கழகம்

கிண்டி,சென்னை.

II. வழிகாட்டி

மரு. சுதந்தரன் ஹம்சவர்தினி, எம்.டி,

இணை பேராசிரியர்,

குருதியேற்று துறை

தமிழ்நாடு டாக்டர் எம்.ஜி.ஆர் மருத்துவ பல்கலைக்கழகம்

கிண்டி,சென்னை.

III. இணை வழிகாட்டி:

மரு. மார்கரெட், எம்டி., டி.எம்.,

பேராசிரியர் மற்றும் தலைவர்,

குருதியியல் துறை ,

ராஜீவ் காந்தி பொது மருத்துவமனை,

சென்னை.

GRADING OF AGGLUTINATION

Figure 2

0/NEGATIVE: Well-defined pellet of non-agglutinated red blood cells at the bottom of the gel column and no visible agglutinated cells in the rest of the gel column.

w+: Barely visible small-sized clumps of agglutinated cells in the lower part of the gel column and a pellet of unagglutinated cells at the bottom.

1+: Some small-sized clumps of agglutinated cells most frequently in the lower half of the gel column. A small pellet may also be observed at the bottom of the gel column.

2+: Small or medium-sized clumps of agglutinated cells throughout the gel column. A few unagglutinated cells may be visible at the bottom of the gel column.

3+: Medium-sized clumps of agglutinated cells in the upper half of the gel column.

4+: A well-defined band of agglutinated red blood cells in the top part gel column. A few agglutinated cells may be visible below the band.

Mf: Mixed-field. A band of red blood cells at the top part of the gel or dispersed throughout the gel column, and a pellet in the bottom as a negative result.

H: Hemolysis in the microtube with very few or no red blood cells in the gel column. Report if hemolysis is present in the microtube but not in the sample.

MONOSPECIFIC DAT

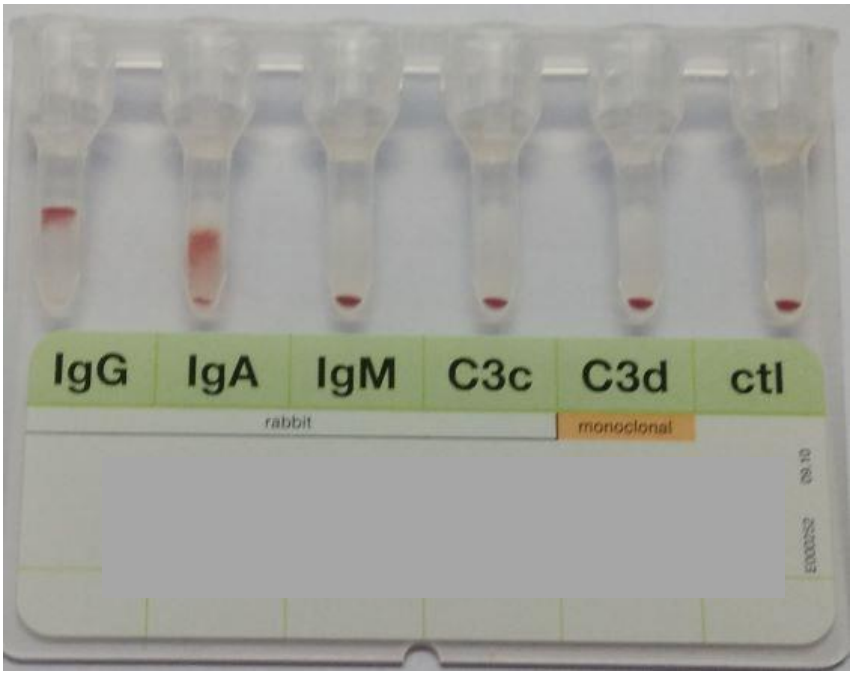

Figure 3

IgG SUBCLASS

Figure 4

ELUATE TESTING

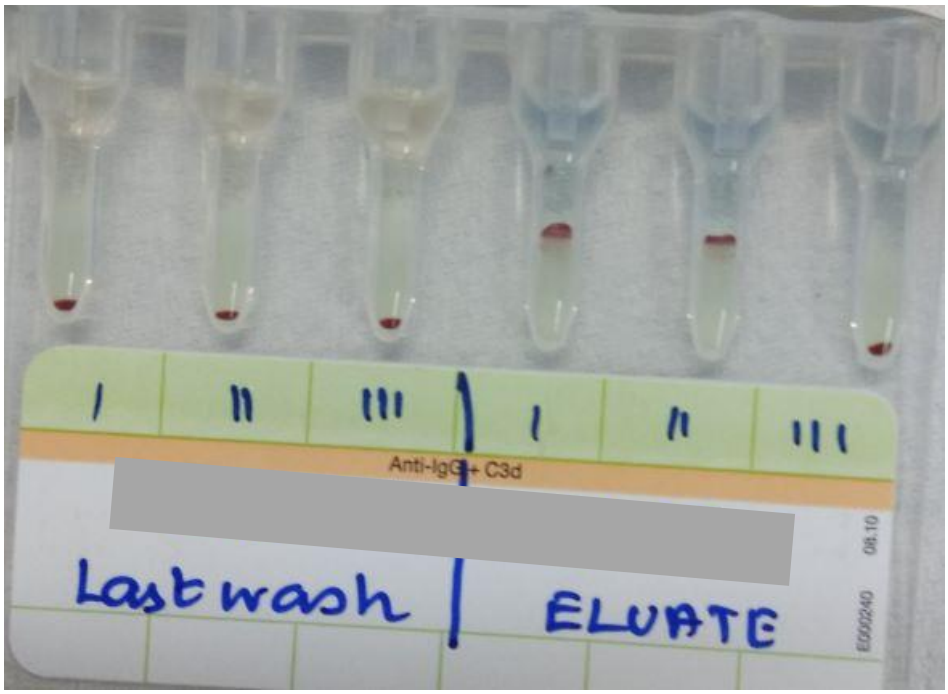

Figure 7

ELUATE TESTING II

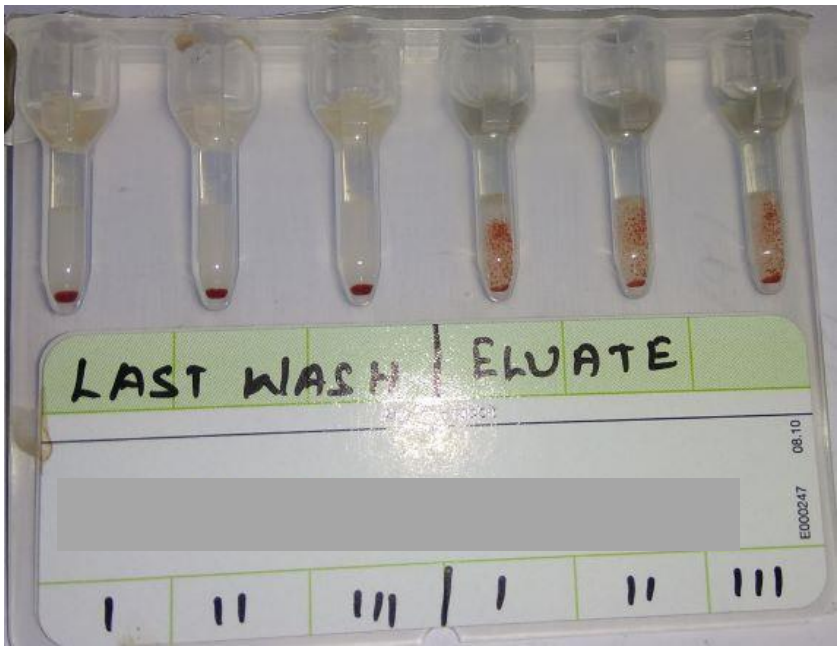

Figure 8

GLYCINE ACID ELUTION KIT

Figure 5

GLYCINE ACID EDTA KIT

Figure 6

RBC Elution

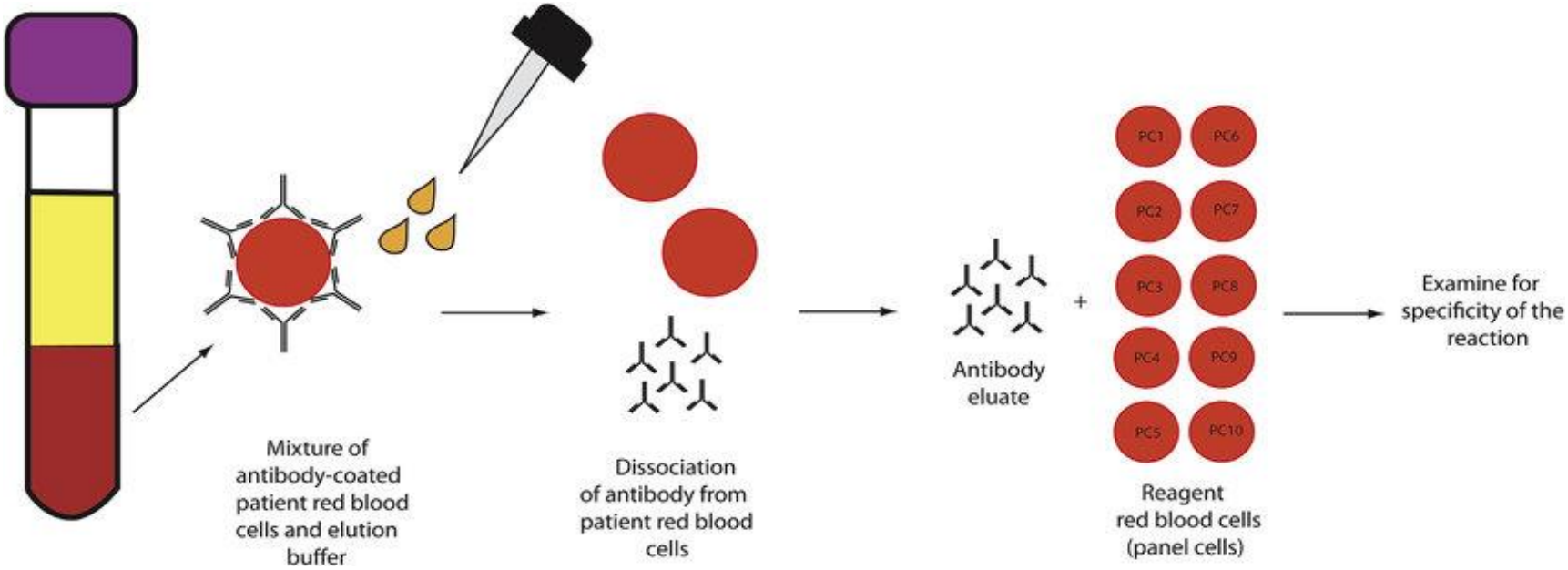

Figure 1

Picture Courtesy: Hendrickson & Tormey

Comparison Chart of DAT positive AIHA cases

Parameters		Present study	Petz LD	Dacie JV	Wheeler et al	Sokol et al	SS Das et al	Wikman	Lai et al
Distribution of AIHA	Primary	59%				57%	44%		
	Secondary	41%				43%	56%		
Age	Primary	>40 yrs 58%	>40 yrs 73%						
	Secondary	<40 yrs 57%							
Type of AIHA	wAIHA	74%	70.3%			62.8%			
	Cold AIHA	17%	15.6%			29.3			
	Mixed	9%				6.9%			
M:F ratio	Primary	1:2.2		F:58%	1.27:1	1.15 to 1:1.33	1:3.3		
	Secondary	1:1.8							
Severity of AIHA	Primary	60%					52.9%	yes	
	Secondary	40%					47.1%		
DAT vs severe hemolysis	>2+	75%			86%		70%	42%	
	<2+	10%			14%		15%	0%	
Distribution of autoAbs	IgG & other Abs	50%		51%		36.9%		59%	55.8%
	IgG alone	32%		36%				34%	44.2%
	C3d alone	18%		11%				9%	
Multiple autoAbs vs severe Hemolysis	Multiple Abs	80%				yes	83.3%		
	Solitary IgG	15%					22.6%		
Auto & Allo Abs		12%	yes						
Distribution of IgG sub type of autoAbs	IgG1	40%		42%		63.1%	37.2%		59%
	IgG1&IgG3	49%		14%		34.9%	9.3%		33.3%
	IgG3	11%		-					7.7%
IgG Sub type of vs severe Hemolysis	IgG1	7/8 (87%)					59%		
	IgG1&IgG3	10/11 (90%)							
	IgG3	1/3 (25%)							

Comparison Chart of DAT positive AIHA cases

Parameters		Present study	Petz LD	Johnston FM study	Dacie JV	Katharia R et al	Yazer MH et al	Wikman A et al	Laine and Beattie,	SS das et al
Elution	Acid Elution	yes					yes			
	Gly Acid EDTA	yes				yes				
Reactivity of Eluate	Reactive	71%	Yes	37%	yes		62.3%			
	nonreactive	29%		63%			37.7%			
DAT Strength	Negative	19 (62%)				37%				
	decreased	9 (38%)				63%				
AutoAb specificity	Anti-e	21%	Anti-e (M/c)		yes					
	Anti-I	6/6 (100%)	91%							
Auto & allo Ab		12%	Yes					28%	37.6%	37.6%
Thermal Amplitude	> 37˚C	25 (74%)								81.3%
	4˚C to 37˚C	6 (17%)								16.3%
	4˚C to 22˚C	3 (9%)								

Comparison Chart of DAT positive HDFN cases

Parameters		Present Study	Dharmesh et al	Frankowska et al	Pollock	Nance et al	Greco et al	Mustafa Aydin et al
Etiology	Rh HDFN	80%	55.5%					
	ABO	14%	27.3%					
	others	6%	17.3%					
Strength of DAT vs hemolysis	<2+	16 (76%)						yes
	>2+	5 (24%)						
Specificity	IgG only	21/21			98/98			
IgG subtype in HDFN	IgG1	52%		34%	32.6%			
	IgG1&3	29%		32%	64.2%			
	IgG3	14%		10%	3.1%			
	No IgG1&/3	5%		7.7%				
IgG subclass vs severity of HDFN	IgG1	81% Tt				56% Tt		
	IgG1&3	100% Tt				90% Tt		
	IgG3	33% Tt				47% Tt		
Elution	IgG1&3	20					34	
	IgG3	1					1	

Comparison Chart of DAT positive DHTR cases

Parameters		Present study	Pineda et al	Erwin Strobel	Salama	Ness et al	Johnston et al
Strength of DAT	1+	1					
	2+	1					
Features Of Hemolysis	HGB	<8gm/dl	anemia	anemia			
	DSB	4mg/dl	Elevated Bilirubin	Mild jaundice			
	LDH	>500IU/ml					
Antibody Distribution	IgG & C3d	2/2			25/26	5/13	
Elution	Glycine Acid	2/2			25/26		7/7
Antibody specificity	Serum & Eluate	1				12	5
	Only Eluate	1				1	2

PLAGIARISM CERTIFICATE - I

Urkund Analysis Result

Analysed Document:	DAT Thesis.docx (D42758084)
Submitted:	10/19/2018 7:13:00 AM
Submitted By:	kavitharaja2006@gmail.com
Significance:	1 %

Sources included in the report:

avani+5_red_7.docx (D34595396)
https://oncohemakey.com/autoimmune-hemolytic-anemia-2/

Instances where selected sources appear:

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